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1: Int J Syst Bacteriol. 1991 Jan;41(1):31-8. Related Articles, Links

Phylogeny of Helicobacter felis sp. nov., Helicobacter mustelae, and related bacteria.

Paster BJ, Lee A, Fox JG, Dewhirst FE, Tordoff LA, Fraser GJ, O'Rourke JL, Taylor NS, Ferrero R.

Forsyth Dental Center, Boston, Massachusetts 02115.

Strain CS1T (T = type strain) is a gram-negative, microaerophilic, urease-positive, spiral-shaped bacterium that was isolated from the gastric mucosa of a cat. Additional strains which possessed biochemical and ultrastructural characteristics similar to those of strain CS1T were isolated from the gastric mucosa of cats and dogs. The guanine-plus-cytosine content of the DNA of strain CS1T was 42.5 mol%. The 16S rRNA sequences of strain CS1T, strain DS3 (a spiral-shaped isolate from a dog), and Helicobacter mustelae were determined by direct RNA sequencing, using a modified Sanger method. These sequences were compared with the 16S rRNA sequences of Helicobacter pylori, "Flexispira rappini," Wolinella succinogenes, and 11 species of campylobacters. A dendrogram was constructed based upon sequence similarities. Strains CS1T and DS3 were very closely related (level of similarity, 99.3%). Two major phylogenetic groups were formed; one group consisted of strains CS1T and DS3, H. mustelae, H. pylori, "F. rappini," and W. succinogenes, and the other group contained the true campylobacters. The average level of similarity between members of these two groups was 84.9%. Within the first group, strains CS1T and DS3, H. pylori, and H. mustelae formed a cluster of organisms with an interspecies similarity level of 94.5%. The phylogenetic positions of W. succinogenes and "F. rappini" were just outside this cluster. On the basis of the results of this study, we believe that strains CS1T (= ATCC 49179T) and DS3 represent a new species of the genus Helicobacter, for which we propose the name Helicobacter felis.

Gastroenterology. 1997 Oct;113(4):1118-28. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Gastritis in urease-immunized mice after Helicobacter felis challenge may be due to residual bacteria.

Ermak TH, Ding R, Ekstein B, Hill J, Myers GA, Lee CK, Pappo J, Kleanthous HK, Mònath TP.

OraVax, Inc., Cambridge, Massachusetts, USA.

BACKGROUND & AIMS: Oral immunization with recombinant Helicobacter pylori urease (rUre) coadministered with a mucosal adjuvant protects mice against challenge with Helicobacter felis. In this study, the duration of protection and gastritis after challenge were characterized at sequential time intervals up to 1 year. METHODS: Outbred Swiss-Webster mice were orally immunized with rUre plus adjuvant and examined for the presence of H. felis infection and leukocyte infiltration into the gastric mucosa. RESULTS: When defined by gastric urease activity, 70%-95% of rUre-immunized mice were protected for between 2 and 57 weeks. Challenge with H. felis increased the inflammatory response in the gastric mucosa of rUre-immunized mice, which also had elevated CD4+ and CD8+ T cells. The CD8+ cells represented a population of gastric intraepithelial cells, which expressed the mucosal alpha E-integrin. Epithelial changes consisting of parietal cell loss and hyperplasia of the epithelium occurred in approximately 20% of the mice. Antimicrobial triple therapy significantly decreased the degree of gastritis and epithelial alteration in the stomach. CONCLUSIONS: These results indicate that oral immunization of mice with rUre produces a long-lasting inhibition of H. felis infection but that residual bacteria may produce a persistent lymphocytic infiltration under these experimental conditions.

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Urease-Specific Monoclonal Antibodies Prevent *Helicobacter felis* Infection in Mice

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Experiments were performed to determine the antigenic specificity of a monoclonal antibody (immunoglobulin A [IgA] 71) previously demonstrated to neutralize the ability of *Helicobacter felis* to colonize mice. Immunoprecipitation of radiolabeled *H. felis* outer membrane proteins with IgA 71 revealed specificity for a 62-kDa protein. Another of our monoclonal antibodies, IgG 40, precipitated a protein of similar molecular weight. IgA 71 but not IgG 40 also precipitated purified recombinant *H. pylori* urease. The antigenic specificity of both antibodies was confirmed to be urease by the ability of each to select *Escherichia coli* clones expressing the *H. felis* urease genes. The two antibodies were shown to bind nonoverlapping epitopes in a competition enzyme-linked immunosorbent assay. Both IgA 71 and IgG 40 could effectively neutralize *H. felis* infectivity by incubating the bacteria with the antibodies prior to oral administration to naive mice. The mechanism of protection does not appear to be inhibition of urease activity, as IgA 71 does not inhibit the conversion of urea to ammonia by *H. pylori* urease in vitro. These results support a protective role for the secretory humoral immune response in *Helicobacter* immunity and provide further evidence that the urease enzyme can serve as a protective antigen.

Helicobacter pylori is a small, spiral, microaerophilic, gram-negative bacterium that colonizes the human upper gastrointestinal tract, primarily the stomach (27). It is now recognized as a significant gastrointestinal pathogen for its etiologic role in nonautoimmune type B gastritis, peptic ulcer disease (21), and mucosa-associated lymphoid tissue gastric lymphoma (13, 29). Additionally, there is an epidemiological association with gastric carcinoma incidence (7).

Current strategies aimed at eradication of *H. pylori* from patients with duodenal ulcers rely on triple antimicrobial therapy which includes a bismuth salt in combination with two antibiotics or omeprazole plus an antibiotic. These treatments must be administered multiple times a day for up to a month. However, despite the susceptibility of *H. pylori* to many antibiotics in vitro, antimicrobial treatment in vivo can be ineffective and even when successful may not prevent reinfection. Therefore, we have been investigating immunization and immunotherapy as a means of prevention and treatment of infection with *H. pylori* and its sequelae.

It has been demonstrated that specific secretory immunoglobulin A (sIgA) can prevent colonization by bacterial pathogens or infection by a viral pathogen at mucosal surfaces (18, 20, 28). Such an sIgA response has been generated at the gastric epithelia of both mice and ferrets. We have demonstrated in both mice and ferrets that multiple oral immunizations with *H. pylori* lysates, in combination with the mucosal adjuvant cholera toxin, generate an *H. pylori*-specific sIgA response in the gastrointestinal tract (4). Subsequently, we have described a similar vaccination protocol using *H. felis* lysates in a germ-free mouse model that results in protection from challenge with infectious *H. felis* (3). In the same study, *H. felis* preincubated with a specific monoclonal antibody (MAb), IgA 71, did not colonize mice. The identification of the antigen(s) recognized by this and other protective MAbs and elucidation of the mechanism(s) by which antibody neutralizes the bacteria would facilitate the development of a subunit vaccine for *Helicobacter* infections. Therefore, the objectives of this study were to define the antigenic specificity of this protective MAb and to analyze the protective potential of other *H. felis*-specific MAbs.

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MATERIALS AND METHODS

Mice. Six- to eight-week-old, germ-free, outbred Swiss Webster mice purchased from Taconic (Germantown, N.Y.) were housed in microisolator cages under germ-free conditions throughout the immunization protocol. Animals were fed autoclaved laboratory chow and water ad libitum. The animals were maintained in a germ-free state, as judged from our inability to culture any bacteria other than those experimentally introduced. The Case Western Reserve University animal facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Bacteria. A bacterial strain isolated originally from a feline gastric biopsy specimen was identified as *H. felis* on the basis of colony morphology, Gram stain, and the production of urease, catalase, and oxidase. *H. felis* was grown on Columbia agar (Difco, Detroit, Mich.) containing 7% horse blood under microaerophilic conditions (5% O_2, 10% CO_2) at 37°C for 96 h. Bacteria were stored at −70°C in 0.1 M phosphate (pH 7.2)–0.9% phosphate-buffered saline (PBS) with 25% glycerol and 25% heat-inactivated fetal calf serum.

OMPs. *H. felis* from 200 confluent plates was harvested in 1 ml of 50 mM Tris (pH 7.8)–1 mM EDTA. One milligram each of DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) were added immediately prior to sonication. Iced bacteria were sonicated in four 30-s bursts with 30-s intervals. Unbroken cells were removed by centrifugation using a Sorvall SS-34 rotor at 9,000 rpm and 4°C for 30 min. Outer membrane proteins (OMPs) were recovered by centrifugation of the supernatant in a Beckman 50Ti rotor at 45,000 rpm for 1 h at 4°C. The pellet was resuspended in 2 ml of 2% *n*-lauroyl sarcosine (Sigma) and allowed to sit at room temperature for 20 min. The solution was spun again at 40,000 rpm for 1 h at 4°C using the Beckman 50Ti rotor, and the pellet was washed three times by resuspension in 1% *n*-lauroyl sarcosine and spinning at 40,000 rpm for 1 h at 4°C using the Beckman 50Ti rotor. The final pellet was resuspended in 50 mM phosphate buffer (pH 7.0) and frozen at −70°C. The concentration of the final OMP solution was determined by the Lowry assay (15).

Urease. Recombinant *H. pylori* urease apoenzyme was purified from *Escherichia coli* ORV154 as previously described (12, 14). Briefly, whole bacteria were harvested by centrifugation, lysed, and subjected to ultrafiltration. The filtrate was absorbed onto DEAE-Sepharose (Pharmacia Biotec Inc., Piscataway, N.J.).

Urease was eluted from DEAE with 150 mM NaCl and used for immunoprecipitations (see below). Native *H. pylori* urease for the enzyme inhibition assay was purified by harvesting bacteria from blood agar plates, lysis, and DEAE binding. Eluted urease was applied to Sephacryl 300 (Pharmacia). Fractions containing urease activity were pooled, bound to Mono Q resin (Pharmacia), and eluted with a 0 to 1 M NaCl gradient.

MAb production. *H. felis*-specific MAbs were prepared as previously described (3) by a modification of the procedure described by Mazanec et al. (18). Briefly, BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by gastric intubation four times over a 6-week period with 2 mg of *H. felis* sonic extract. The first three doses included 10 μg of cholera toxin (Sigma), and the final dose was given without cholera toxin but was accompanied by an intravenous boost of 2 mg of *H. felis* sonic extract. Three days following the final immunization, mice were sacrificed, and their spleens cells were hybridized to SP2/0 myeloma cells. Clones were obtained by limiting dilution and screened for anti-*H. felis*-specific antibodies by enzyme-linked immunosorbent assay (ELISA) using *H. felis* OMP antigen. Stable antibody secretors were injected intraperitoneally into pristane-primed BALB/c mice, and the ascitic fluid was harvested and clarified.

ELISA. Ascites fluids were assayed for anti-*H. felis* OMP specificity as follows. Microtiter plates containing, per well, 100 μl of *H. felis* OMP (10 μg/ml) in PBS were placed in humid chambers at 4°C overnight. Plates were emptied by inversion and blocked for 1 h at room temperature with 200 μl of 1% bovine serum albumin (BSA) per well. Plates were washed three times between each subsequent step with 0.1% BSA in PBS. Ascites fluids (100 μl per well) in half-log dilutions were added and incubated for 1 h at room temperature. Antibodies specific to OMP were detected by incubating either a goat anti-mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala.)- or goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.)-alkaline phosphatase conjugate (100 μl per well) for a further hour at room temperature. After the plates were developed with 100 μl of *p*-nitrophenylphosphate (1 mg/ml) per well, the change in optical density at 410 nm was measured and recorded on an MR 700 microtiter plate reader (Dynatech Laboratories, Inc., Chantilly, Va.).

Competition ELISAs were performed between IgA 71 and IgG 40 to determine if IgG 40 bound to epitopes distinct from or overlapping with IgA 71. Preliminary ELISAs showed that a 10^3 dilution of IgG 40 ascites fluid resulted in subsaturation levels of alkaline phosphatase activity. Therefore, this dilution was chosen for use in the competition. One hundred microliters of IgG 40 ascites fluid diluted 10^3 was combined with an equal volume of IgA 71 ascites fluid diluted in half-log increments prior to the 1-h incubation in *H. felis* OMP-coated microtiter wells. The remainder of the assay was performed as described above, using an anti-IgG-alkaline phosphatase conjugate. In a control experiment, IgG 40 was placed in competition with an irrelevant IgA ascites fluid, Sv255 (specific for Sendai virus), for binding to OMP. In the reciprocal competition, a 10^3 dilution of IgA 71 was combined with various dilutions of IgG 40. In this case, binding of IgA 71 was measured by using an IgA-specific conjugate. Results of the competition ELISA were compared with the activities of the MAbs tested in the absence of a competing antibody.

Urease inhibition assay. Inhibition of urease activity by MAb IgA 71 was determined by comparison of the rate of release of ammonia from urea in the presence of various concentrations of IgA 71. Purified *H. pylori* urease (0.1 μg) was incubated at 37°C for 30 min in the presence of 0, 0.1, 1.0, or 10 μg of IgA 71 in 96-well microtiter plates. Rapid urea test broth (100 μl) was added to each mixture, and the incubation continued at room temperature. The color development was monitored at 5-min intervals at 550 nm, using a microtiter plate reader.

Radiolabeling of proteins. *H. felis* OMP and purified *H. pylori* urease were radiolabeled with $Na^{125}I$ by using lactoperoxidase (26). Briefly, 10 μg of protein was combined with 30 mU of lactoperoxidase, 1.0 mCi of $Na^{125}I$, and 10 μl of 0.00042% H_2O_2 for 30 s at room temperature. The reaction was stopped by the addition of 100 μl of 0.1% BSA in PBS with 0.05% NaN_3. Labeled proteins were separated from carrier-free ^{125}I by fractionation on a 10-ml Sephadex G-50 (Pharmacia) column. Aliquots from individual fractions were counted in a Beckman 5000 γ counter (Beckman Instruments, Inc., Fullerton, Calif.) and subsequently precipitated with 20% trichloroacetic acid. The fractions corresponding to the aliquots of which greater than 98% of the activity could be precipitated were pooled and saved for use in immunoprecipitation assays.

Coupling antibodies to Sepharose 4B. Sepharose 4B (Pharmacia) was activated according to the manufacturer's instructions. Briefly, 1 g of Sepharose 4B was swollen in 10 ml of H_2O, and the resulting slurry was washed extensively with H_2O. Five grams of the slurry was placed in a beaker on ice and combined with 5 ml of 10% CNBr solution. One molar NaOH was added to the slurry while stirring, and the pH was monitored until a reading of 11 to 11.5 could be maintained for at least 10 min. The activated Sepharose was then washed extensively with 10 volumes of cold 0.1 M $NaHCO_3$. Five milliliters of a 1-mg/ml solution of affinity-pure goat anti-mouse IgA antibodies (Southern Biotechnology) was dialyzed against 0.1 M $NaHCO_3$, combined with 2 g of the activated Sepharose 4B, and then rocked overnight at 4°C. An aliquot of the supernatant was assayed for protein content (15) to determine the efficacy of binding.

Immunoprecipitation. Microcentrifuge tubes were precoated with dilution buffer (0.1% Triton X-100, 0.1% BSA, 0.01 M Tris [pH 8.0], 0.14 M NaCl) for 10 min at room temperature. The tubes were emptied, 10 μl (approximately 10^6 cpm) of ^{125}I-labeled *H. felis* OMP or *H. pylori* urease was aliquoted to each tube, and the volume was brought up to 200 μl with dilution buffer. Ten microliters of ascites fluid was added to each tube, and the contents were rocked for 1.5 h at 4°C. Fifty microliters of goat anti-mouse IgA–Sepharose 4B conjugate (prepared as described above) or protein G-Sepharose (Pharmacia) mixed 1:1 with dilution buffer was added, and the contents were rocked for 1.5 h at 4°C. The tubes were spun at 200 × *g* to pellet the beads and washed twice with dilution buffer, once with 0.01 M Tris (pH 8.0)–0.14 M NaCl, and once with 0.05 M Tris (pH 6.8). Fifty microliters of sodium dodecyl sulfate sample buffer was added to the pellet and heated for 5 min at 100°C. The tubes were gently agitated and spun at 200 × *g* for 2 min, and the supernatants (50 μl) were loaded onto 10% polyacrylamide gels for polyacrylamide gel electrophoresis. Gels were dried on filter paper under vacuum and developed by autoradiography. Controls included tubes without antibody, as well as IgA Sv255 and IgG Sv271 MAbs specific for Sendai virus glycoproteins. Anti-Sendai virus MAbs were a gift from Mary Mazanec, Case Western Reserve University, Cleveland, Ohio.

Passive oral immunization. *H. felis* was harvested from a fresh plate and brought to a concentration of 5 × 10^6 CFU/ml in PBS. Two-milliliter aliquots were combined with 2 ml of ascites fluid containing MAb (IgA 71 anti-*H. felis* OMP, IgG 40 anti-*H. felis* OMP, or IgA 255 anti-Sendai virus) and incubated at 37°C for 30 min. Four hundred microliters (10^6 organisms) was administered to each mouse by gastric intubation. All mice received an additional 200 μl of ascites fluid at 4, 8, and 24 h. Mice were necropsied on day 9, and the gastric tissue was examined for *H. felis* colonization by the urea broth assay and culture methods described below.

Urease detection assay. Biopsy specimens (2 by 2 mm) were taken from the gastric antrum, placed in 0.5 ml of Stuart's urease test broth (25), and incubated at room temperature. *H. felis* colonization was confirmed by a change in broth color from orange to red. Tubes were observed over a 24-h period.

Culture. Gastric antral biopsy specimens were homogenized in 200 μl of Columbia broth and plated on Columbia blood agar with 7% horse blood. Plates were incubated at 37°C for 96 h under microaerophilic conditions. Colonies were confirmed as *H. felis* on the basis of morphology, Gram stain, and the production of urease, catalase, and oxidase.

Antibody screening of *H. felis* expression library. Genomic DNA was isolated from *H. felis* CS1 (a gift from James Fox, Massachusetts Institute of Technology, Cambridge) by the Marmur technique (16). This DNA was sent to Stratagene (La Jolla, Calif.), where it was used to construct an *H. felis* genomic DNA expression library containing 6- to 10-kb inserts in lambda ZAP II. Five thousand plaques from five 150-mm-diameter plates were replicated onto duplicate nitrocellulose filters soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Replicate filters were screened for antigen-producing recombinants by immunoblotting using MAbs as probes. Briefly, filters were blocked in 1% BSA in 20 mM Tris-buffered saline (pH 7.5) at 4°C overnight and then incubated with either MAb IgA 71 or MAb IgG 40 diluted 1:2,000 in blocking buffer for 1 h at room temperature. After filters were washed in Tris-buffered saline with 0.05% Tween 20 (Sigma), antibody binding was detected by soaking the filters for 1 h at room temperature in alkaline phosphatase-conjugated goat anti-mouse isotype antibodies (see ELISA methods) diluted 1:2,000 in blocking buffer. After a further wash, all phosphatase activity was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (Bio-Rad Laboratories, Hercules, Calif.) as a precipitating substrate. Positive plaques were picked and stored in 500 μl of SM buffer (0.1 M NaCl, 5 mM $MgSO_4$, 50 mM Tris [pH 7.5]) and 50 μl of chloroform.

Recovery of plasmids from lambda phage vectors. Plasmids were rescued from positive lambda phage recombinants as recommended by Stratagene. Briefly, 200 μl of Stratagene *E. coli* SOLR cells (optical density at 600 nm of 1.0) was combined with 200 μl of recombinant phage stock (from SM buffer storage; see above) and 1 μl (10^{10}/ml) of Stratagene ExAssist helper phage. After incubation at 37°C for 15 min, 5 ml of 2× YT medium (16 g of Bacto Tryptone, 10 g of yeast extract, and 10 g of NaCl per liter) was added, and the culture was incubated at 37°C for 3 h with shaking. Cultures were heated at 70°C for 20 min to kill the bacteria. The phage were separated from particulate residue by centrifugation at 6,000 rpm in a Sorvall SS-34 rotor for 5 min at room temperature. Ten microliters of supernatant containing the rescued plasmid in filamentous phage particles was incubated with 200 μl of Stratagene *E. coli* XL1-Blue cells (optical density at 600 nm of 1.0) at 37°C for 15 min and plated on LB agar containing ampicillin (100 μg/ml) for selection of *E. coli* harboring the rescued plasmids.

DNA hybridization. Restriction digests of purified plasmids were resolved on 1% agarose gels. DNA was transferred to GeneScreen membranes (Du Pont NEN Research Products, Boston, Mass.) by the Southern method (24) and hybridized to *ure*B-specific fluorescein-labeled oligonucleotides at 42°C for 2 h. Hybridization was detected with Amersham's enhanced chemiluminescence system (Amersham Corp., Arlington Heights, Ill.). The same oligonucleotides were used as primers in the sequencing reactions listed below.

Sequencing. Sequencing was accomplished by the dideoxy-chain termination method (23), using Sequenase (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's instructions. The oligonucleotides used as primers included a T3 promoter primer (5'ATTAACCCTCACTAAAG3'), a T7 promoter primer (5'AATACGACTCACTATAG3'), and an *H. pylori ureB* primer (HpURE1; 5'ATGAAAAAGATTAGCAGAAAAGAATATGTTTCTATG3').

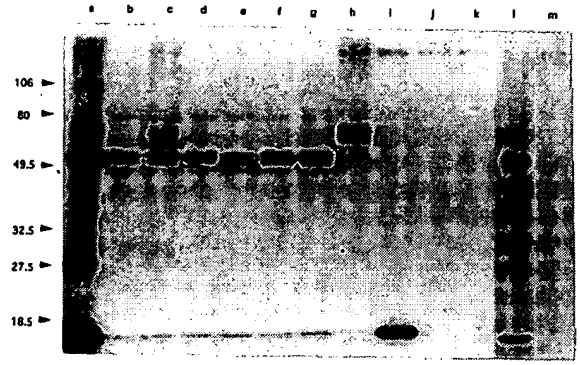

FIG. 1. Immunoprecipitation of radiolabeled *H. felis* OMPs with anti-*H. felis* MAbs. Goat anti-mouse IgA–Sepharose was used as a precipitant for lanes b to g and l. Protein G-Sepharose was used as a precipitant for lanes h to k and m. Precipitated antigens were resolved on a 10% polyacrylamide gel. Lanes: a, labeled OMPs; b, IgA 34; c, IgA 71; d, IgA 109; e, IgA 251; f, IgA 395; g, IgA 255 (anti-Sendai virus); h, IgG 40; i, IgG 50; j, IgG 84; k, IgG 271 (anti-Sendai virus); l, no antibody; m, no antibody. Sizes are indicated in kilodaltons.

Plasmid analysis and subcloning. Restriction endonucleases and all other enzymes used for the manipulation of DNA were purchased from Gibco-BRL (Gaithersburg, Md.). All common DNA manipulations were performed as described by Sambrook et al. (22). Plasmid DNA was isolated from cells lysed by boiling (11), and transformations were performed by using a one-step transformation and storage solution (1a).

Antibody screening of bacterial colonies. Colonies of bacteria harboring plasmid pBluescript II SK(−) (Stratagene) or pTGB1 were grown on nitrocellulose filters and screened with MAbs for the production of antigen as described by Sambrook et al. (22). Briefly, the nitrocellulose filters were exposed to chloroform vapors for 15 min and then incubated in lysis buffer (0.1 M Tris [pH 7.8], 0.15 M NaCl, 5 mM $MgCl_2$, 1.5% BSA, 1 mg of pancreatic DNase I per ml, 400 μg of lysozyme per ml) while shaking on an orbital shaker overnight at room temperature. Membranes were washed three times for 30 min each in 10 mM Tris (pH 8.0)–0.15 M NaCl–0.05% Tween 20 (TNT) and were then blocked for 30 min in 20% fetal calf serum in TNT. Filters were subsequently incubated for 4 h in MAb ascites fluid diluted 1:2,000 in blocking buffer, washed three times for 10 min each in TNT containing 0.1% BSA, and then incubated in goat anti-mouse isotype-alkaline phosphatase conjugates (see procedures for library screening) before being washed again and developed with BCIP and nitroblue tetrazolium.

Statistical analysis. The presence or absence of experimental infection among groups of mice was evaluated by χ^2 analysis.

RESULTS

Immunoprecipitation of *H. felis* OMPs with MAbs. We previously reported that incubation of infectious *H. felis* with an anti-*H. felis* monoclonal IgA ascites fluid in vitro effectively neutralized the ability of the bacteria to colonize the stomachs of germ-free mice (3). The protective MAb IgA 71 failed to bind specifically to any *H. felis* proteins in Western blots (immunoblots) (not shown). Therefore, IgA 71 and seven other anti-*H. felis* MAbs developed in our laboratory were used to immunoprecipitate radiolabeled *H. felis* OMPs in a further attempt to determine their antigenic specificities. A protein of 62 kDa was precipitated by MAb IgA 71 (Fig. 1, lane c), and MAb IgG 40 precipitated a protein of similar molecular weight (lane h). None of the other six MAbs specifically precipitated any OMPs in this molecular weight range. Although other protein bands appeared in all lanes, these were attributed to association of OMPs with the goat anti-mouse IgA–Sepharose (lanes b to g and l) or protein G-Sepharose (lanes h to k and m) used to immunoprecipitate the immune complexes. This result was verified by the banding pattern achieved when radiolabeled OMPs were combined with goat anti-mouse IgA–Sepharose (lane l) or protein G Sepharose (lane m) in the absence of antibody.

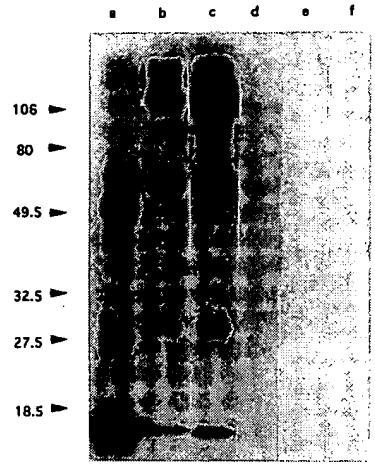

FIG. 2. Immunoprecipitation of radiolabeled *H. pylori* urease. Goat anti-mouse IgA–Sepharose was used as a precipitant for lanes c and d. Protein G-Sepharose was used as a precipitant for lanes e and f. Lanes: a, *H. felis* OMPs; b, *H. pylori* urease; c, IgA 71; d, IgA 255 (anti-Sendai virus); e, IgG 40; f, IgG 271 (anti-Sendai virus). Sizes are indicated in kilodaltons.

Immunoprecipitation of *H. pylori* urease with MAb IgA 71. The results from the immunoprecipitation with *H. felis* OMPs suggested that both IgA 71 and IgG 40 bind to the large subunit of *H. felis* urease, which has a molecular mass of 62 kDa. Amino acid sequence analysis of the N terminus of the gel-purified protein was inconclusive. Since MAb IgA 71 binds to both *H. felis* and *H. pylori* OMPs in ELISA (not shown) and since the deduced *H. pylori* and *H. felis* urease amino acid sequences are highly homologous (8), we investigated whether IgA 71 and IgG 40 would immunoprecipitate recombinant *H. pylori* urease. Urease apoenzyme prepared under nondenaturing conditions was precipitated by IgA 71 (Fig. 2, lane c) but not by IgG 40 (Fig. 2, lane e). This result confirmed the recognition of *Helicobacter* urease proteins by IgA 71 and suggested that IgG 40 either recognizes a different *H. felis* OMP of approximately 62 kDa or may bind to an epitope of *H. felis* urease which is not shared by *H. pylori* urease.

Screening of an *H. felis* expression library with MAbs. To clarify the antigenic specificities of IgA 71 and IgG 40, we next used these antibodies as probes to screen an *H. felis* lambda phage expression library. Ten plaques representing four distinct clones were identified when 5,000 plaques were probed with IgA 71. All IgA 71-positive plaques also reacted with IgG 40. The plasmids from all four positive clones were rescued by in vivo excision. Southern blot analysis revealed that all of the plasmids could be hybridized by a synthetic oligonucleotide corresponding to the first 36 nucleotides of the 5′ end of the *H. pylori ureB* gene (2) (data not shown). The predicted amino acid sequence derived from a partial nucleotide sequence from one of the clones, pTGB1, is shown in Fig. 3 and compared with the published sequences of both *H. pylori* and *H. felis ureB* (2, 8). The first 73 amino acids share 79.5% identity with the *H. pylori ureB* sequence and 97.3% identity with the recently reported sequence of *H. felis ureB* from strain ATCC 49179 (8).

pTGB1 contained an *Eco*RI insert of 5.6 kb which when further analyzed by restriction endonuclease digestion mapping and Southern blotting experiments was found to encode both *ureA* and *ureB*. The presence of restriction fragments identical to those mapped for *ureA* and *ureB* by Ferrero and Labigne (8) strengthened this conclusion. Immunoblots of col-

```
                      10        20        30        40
H. pylori ureB        MKKISRKEYVSMYGPTTGDKVRLGDTDLIAEVEHDYTIYG
IgA 71 ligand         ---------A---------R---------L-----C-T--
H. felis ureB         ---------------------R---------L-----C-T--

                      50        60        70
H. pylori ureB        EELKFGGGKTLREGMSQSNNPSKEELDLIITNA
IgA 71 ligand         --I--------I-D-- -T-S--SY----VL---
H. felis ureB         --I--------I-D----T-S--SY----VL---
```

FIG. 3. Predicted amino acid sequence of DNA from an expression library clone selected with the protective MAb IgA 71 compared with the 5′ ends of the *ureB* genes from *H. pylori* and *H. felis*.

onies performed with both IgA 71 and IgG 40 resulted in the recognition of *E. coli* containing pTGB1 but not to *E. coli* containing vector DNA with no insert. These results confirm that the ligand for both IgA 71 and IgG 40 is urease and when combined with the OMP immunoprecipitation data (Fig. 1) strongly suggest that the *H. felis ureB* protein contains the respective epitopes.

MAbs IgA 71 and IgG 40 bind distinct epitopes. To demonstrate that these two antibodies do indeed recognize distinct *H. felis* urease epitopes as suggested by the failure of IgG 40 to immunoprecipitate *H. pylori* urease, competitive ELISAs were performed. In these assays, we took advantage of the fact that each antibody could be recognized by a distinct isotype-specific second antibody, obviating the need to label one or the other of the primary MAbs. In this competitive ELISA, increasing doses of IgG 40 failed to displace a fixed, subsaturating amount of IgA 71 (Fig. 4). Similar results were obtained in the reciprocal assay in which a fixed amount of IgG 40 was placed in competition with increasing amounts of IgA 71 (data not shown).

Passive protection of mice from *H. felis* by MAbs specific for urease. To compare the effectiveness of MAb IgG 40 in neutralizing *H. felis* with that of MAb IgA 71, ascites fluid containing either IgG 40 or IgA 71 was incubated with 10^6 viable *H. felis* cells prior to oral administration to germ-free mice (see Materials and Methods). Control mice received *H. felis* preincubated with an irrelevant anti-Sendai virus IgA ascites fluid. The results of the experiment are shown in Fig. 5. Twelve of the fourteen mice (86%) receiving *H. felis* preincubated with

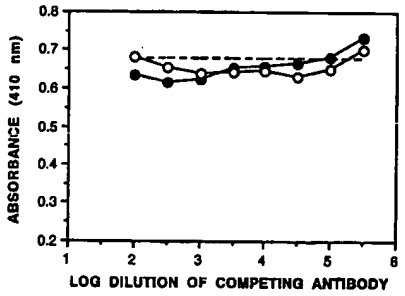

FIG. 4. Effect of IgG 40 on IgA 71 binding to *H. felis* OMPs. A predetermined dilution (10^{-3}) of IgA 71 ascites fluid was combined with serial half-log dilutions of IgG 40 to determine if the two MAbs were specific for overlapping epitopes. Solid circles represent the detectable IgA when IgG 40 is used as an inhibitor of IgA 71. Open circles represent the amount of IgA detected when an irrelevant IgG, Sv271, is used to compete with IgA 71. The line without symbols represents the signal generated by a 10^{-3} dilution of IgA 71 ascites fluid in the absence of an inhibitor.

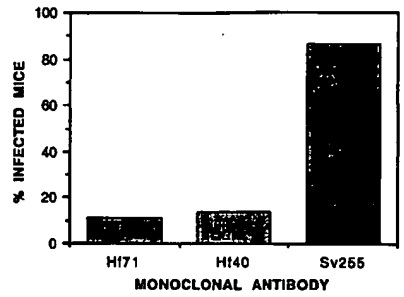

FIG. 5. Protection of germ-free mice from *H. felis* infection by incubation of bacteria with MAb ascites fluid prior to oral inoculation of mice. Groups of 9, 14, and 14 mice were used for MAbs IgA 71, IgG 40, and IgA Sv255, respectively.

irrelevant ascites fluid were colonized by *H. felis*, while only one of the nine mice (11%) receiving *H. felis* preincubated with IgA 71 was infected ($P = 0.002$). These results provide additional support to our previous findings that IgA 71 could protect mice from infection (3). Only two of the fourteen mice (14%) receiving *H. felis* in combination with IgG 40 were infected ($P = 0.0004$). Thus, both IgA 71 and IgG 40 were able to passively protect mice from *H. felis* infection.

MAb IgA 71 does not prevent colonization by inhibiting urease activity. Since IgA 71 precipitates *H. pylori* urease, we tested its ability to inhibit urease activity in a urea hydrolysis assay. Increasing amounts of IgA 71 failed to inhibit the conversion of urea to ammonia as determined in a colorimetric assay (Fig. 6). Thus, the mechanism by which IgA 71 prevents colonization of mice stomachs does not appear to be by inhibition of urease activity. Although urease activity is not affected by IgA 71, both IgA 71 and IgG 40 do agglutinate *H. felis* at the concentrations used for passive protection. This agglutination did not affect bacterial viability, as the agglutinated *H. felis* continued to grow well in culture.

DISCUSSION

This study provides further evidence that *H. felis*-specific antibodies can prevent colonization of mouse gastric tissue by *H. felis* and demonstrates that both an IgA and an IgG neutralizing MAb are specific for urease. Incubation of *H. felis* with either of our MAbs, IgA 71 or IgG 40, prior to oral administration to mice effectively prevents the ability of the bacteria to colonize the gastric mucosa. When combined with

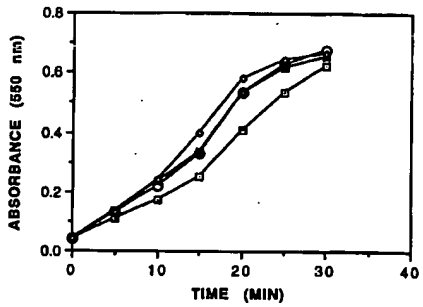

FIG. 6. *H. pylori* urease activity in the presence of MAb IgA 71. Urease holoenzyme (0.1 μg) was combined with 0.0 (open squares), 0.1 (open circles), 1.0 (closed squares), or 10 (open diamonds) μg of IgA 71 and combined with rapid urea broth for 30 min. Urease activity was monitored by increased A_{550}.

our previous report on the induction of protection by active oral immunization, these results indicate the importance of a mucosal, presumably sIgA, immune response in resistance to *Helicobacter* infection.

Urease has been implicated as an important virulence factor in *Helicobacter* pathogenesis (10, 17, 30). It is highly conserved among *Helicobacter* species and functions to convert urea to ammonia, which has been suggested to protect it from the harmful acids of the gastric environment (17). Urease is loosely associated with the bacterial membrane and represents over 5% of the soluble bacterial protein (12). The importance of this enzyme in *Helicobacter* colonization has been underscored by experiments with chemically induced urease-negative mutants of *H. pylori* which fail to colonize barrier-born pigs (6). The present study supports the idea that urease is an important antigen in *Helicobacter* immunity.

Urease-specific sIgA is produced in both humans and mice infected with *Helicobacter* species. While our results may seem contrary to the observation that *Helicobacter* infections persist in the presence of such antibodies, our previous results (3) and those of others (1, 5, 14) have demonstrated that preexisting *Helicobacter*-specific antibodies can prevent colonization despite their inability to eradicate an established infection. Recently, it has been demonstrated that protective immunity against *H. felis* can be achieved in mice by active oral immunization with the urease proteins (9, 19). Our results support these findings, indicating that urease may serve as a possible vaccine candidate and strengthen the notion that protection is antibody mediated.

We have also provided evidence that our infection neutralizing MAbs IgA 71 and IgG 40 are specific for the large subunit of the urease enzyme. Although IgA 71 precipitates both subunits of purified recombinant *H. pylori* urease, it precipitates only the heavy subunit of urease when reacted with *H. felis* OMPs. This is most likely due to a difference in protein preparation. The purified *H. pylori* urease apoenzyme was purified from bacterial supernatants by column chromatography under conditions which would not disrupt subunit association, and therefore both the large and small subunits would be precipitated simultaneously. The *H. felis* OMPs, however, were prepared by repeated washing in *n*-lauroyl sarkosine (see Materials and Methods), and therefore the subunits were dissociated prior to immunoprecipitation. Precipitation of the *H. pylori* urease with IgA 71 is consistent with published sequence analysis of *H. pylori* and *H. felis* urease proteins demonstrating a high degree of conservation (8). However, the specificity of IgG 40 for *H. felis* but not *H. pylori* urease demonstrates that there are species-specific epitopes on the enzyme. Interestingly, Ferrero et al. (9) have reported an increased efficacy of the urease B subunit relative to the A subunit by immunizing mice with various recombinant urease fusion proteins.

Although both antibodies bind to the *ureB* protein, IgA 71, which precipitates *H. pylori* urease, fails to inhibit the enzymatic activity of purified *H. pylori* urease. We postulate that antibody effectiveness is mediated in one of two ways. First, the urease proteins may possess some previously undescribed function, either enzymatic or receptor binding, which is inhibited by the MAbs. Second, the antibody may inhibit colonization by agglutinating the bacteria and promoting clearance by mucus flow. Microscopic examination of *H. felis* incubated with our two OMP-specific MAbs reveals that both are capable of agglutinating the bacteria at the concentrations used in the passive protection studies. It should be noted that several of our other *H. felis*-specific MAbs also agglutinate the bacteria in vitro yet fail to inhibit colonization (unpublished data). It is unlikely that the nature of the ascites fluid plays a role in the protection, as ascites fluids containing irrelevant MAbs fail to prevent colonization.

The ability of IgG 40 to prevent colonization of *H. felis* is interesting in light of the fact that IgG is not present in significant quantities at the mucosa and is more prone to the acid environment of the stomach than IgA. Several explanations may be made for its effectiveness in the present study. First, agglutination is taking place in vitro in a "protein-friendly" environment. The nature of the agglutinated complex might protect the antibodies from the stomach acid when introduced to the mouse. Second, the volume (400 μl) of ascites and bacteria given to the mouse by gastric intubation may be sufficient to change the stomach environment long enough to allow the IgG antibodies to prevent colonization. Presumably the bacteria are displaced by the peristaltic action of the gastric mucosa as immune complexes. Third, the gastric environment of the mouse may be much less hostile than that of the human. In fact the mouse stomach is routinely colonized by other bacterial species. We have also performed experiments in which MAbs are delivered to the stomachs of naive mice prior to challenge with *H. felis*. These mice are not protected from colonization (data not shown), a result most consistent with agglutination being of major importance in passive protection of mice. However, as our experimental system may not have used ideal IgG concentrations, the second two explanations cannot be ruled out. In conclusion, this study provides evidence that the humoral response can be sufficient to prevent colonization and that urease can serve as a protective antigen.

ACKNOWLEDGMENTS

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REFERENCES

1. **Chen, M., A. Lee, and S. Hazell.** 1992. Immunisation against gastric helicobacter infection in a mouse/Helicobacter felis model. Lancet 339:1120–1121.
1a. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA 86:2172–2175.
2. **Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali.** 1989. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res. 18:362.
3. **Czinn, S. J., A. Cai, and J. G. Nedrud.** 1993. Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. Vaccine 11:637–642.
4. **Czinn, S. J., and J. G. Nedrud.** 1991. Oral immunization against *Helicobacter pylori*. Infect. Immun. 59:2359–2363.
5. **Davin, C., A. L. Blum, I. Corthesy-Theulaz, E. Saraga, J.-P. Kraehenbuhl, R. Haas, and P. Michetti.** 1993. *H. pylori* urease elicits protection against *H. felis* infection in mice. Gastroenterology 104:A1035.
6. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. 59:2470–2475.
7. **Eurogast Study Group.** 1993. An international association between *Helicobacter pylori* infection and gastric cancer. Lancet 341:1359–1362.
8. **Ferrero, R. L., and A. Labigne.** 1993. Cloning, expression and sequencing of *Helicobacter felis* urease genes. Mol. Microbiol. 9:323–333.
9. **Ferrero, R. L., J.-M. Thiberge, M. Huerre, and A. Labigne.** 1994. Recombinant antigens prepared from the urease subunits of *Helicobacter* spp.: evidence of protection in a mouse model of gastric infection. Infect. Immun. 62:4981–4989.
10. **Goodwin, C. S., J. A. Armstrong, and B. J. Marshall.** 1986. *Campylobacter pyloridis*, gastritis, and peptic ulceration. J. Clin. Pathol. 39:353–365.
11. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193.
12. **Hu, L.-T., and H. L. T. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. 58:992–998.
13. **Hussell, T., P. G. Isaacson, J. E. Crabtree, and J. Spencer.** 1993. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to *Helicobacter pylori*. Lancet 342:571–574.
14. **Lee, C. K., R. Weltzin, W. D. Thomas, G. Soman, D. M. Houle, A. Lofsky,**

K. M. Georgeakopoulos, A. S. High, T. Tibbetts, and T. P. Monath. 1993. Protection from *Helicobacter* infections by intragastric immunization with recombinant urease, abstr. 13. Program and Abstracts, *Helicobacter pylori*: Basic Mechanisms to Clinical Cure. Falk LTD., Amelia Island, Fla.

15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
17. **Marshall, B. J., L. J. Barret, C. Prakash, R. W. McCallum, and R. L. Guerrant.** 1990. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. Gastroenterology **99**:697–702.
18. **Mazanec, M. B., J. G. Nedrud, and M. E. Lamm.** 1987. Immunoglobulin A monoclonal antibodies protect against Sendai virus. J. Virol. **61**:2624–2626.
19. **Michetti, P., I. Corthesy-Theulez, C. Davin, R. Haas, A.-C. Vaney, M. Heitz, J. Bille, J.-P. Kraehenbuhl, E. Saraga, and A. L. Blum.** 1994. Immunization of Balb/c mice against *Helicobacter felis* infection with *Helicobacter pylori* urease. Gastroenterology **107**:1002–1011.
20. **Michetti, P., M. J. Mahan, J. M. Slaugh, J. J. Mekalanos, and M. R. Neutra.** 1992. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen *Salmonella typhimurium*. Infect. Immun. **60**:1786–1792.
21. **Peterson, W. L.** 1991. *Helicobacter pylori* and peptic ulcer disease. N. Engl. J. Med. **324**:1043–1048.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
25. **Stuart, C., E. Van Stratum, and R. Rustigian.** 1945. Further studies on urease production by proteus and related organisms. J. Bacteriol. **49**:437–444.
26. **Thorell, J. I., and G. B. Johansson.** 1971. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochim. Biophys. Acta **251**:363.
27. **Warren, J. R., and B. J. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i**:1273–1275.
28. **Winner, L. S., III, J. Mack, R. Weltzin, J. J. Mekalanos, J.-P. Kraehenbuhl, and M. R. Neutra.** 1991. New model for analysis of mucosal immunity: intestinal secretion of specific monoclonal immunoglobulin A from hybridoma tumors protects against *Vibrio cholerae* infection. Infect. Immun. **59**: 977–982.
29. **Wotherspoon, A. C., C. Doglioni, T. C. Diss, L. Pan, A. Moschini, M. de Boni, and P. G. Isaaccson.** 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet **342**:575–577.
30. **Xu, J.-K., C. S. Goodwin, M. Cooper, and J. Robinson.** 1990. Intracellular vacualization caused by the urease of *Campylobacter pyloris*. J. Infect. Dis. **161**:1302–1304.

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Recombinant Antigens Prepared from the Urease Subunits of *Helicobacter* spp.: Evidence of Protection in a Mouse Model of Gastric Infection

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Urease is an important virulence factor for gastric *Helicobacter* spp. To elucidate the efficacy of individual urease subunits to act as mucosal immunogens, the genes encoding the respective urease subunits (UreA and UreB) of *Helicobacter pylori* and *Helicobacter felis* were cloned in an expression vector (pMAL) and expressed in *Escherichia coli* cells as translational fusion proteins. The recombinant UreA and UreB proteins were purified by affinity and anion-exchange chromatography techniques and had predicted molecular masses of approximately 68 and 103 kDa, respectively. Western blotting (immunoblotting) studies indicated that the urease components of the fusion proteins were strongly immunogenic and were specifically recognized by polyclonal rabbit anti-*Helicobacter* sp. sera. The fusion proteins (50 μg) were used, in combination with a mucosal adjuvant (cholera toxin), to orogastrically immunize mice against *H. felis* infection. Gastric tissues from *H. felis*-challenged mice were assessed by the biopsy urease test and by histology. In mice immunized with recombinant *H. felis* UreB, 60% of animals (n = 7) were histologically negative for *H. felis* bacteria after challenge at 17 weeks. This compared with 25% (n = 8) for mice immunized with the heterologous *H. pylori* UreB antigen. Neither the homologous nor the heterologous UreA subunit elicited protective responses against *H. felis* infection in mice. The study demonstrated that a recombinant subunit antigen could induce an immunoprotective response against gastric *Helicobacter* infection.

Helicobacter pylori is a gram-negative spiral-shaped bacterium that colonizes the mucus layer associated with gastric-type epithelium in humans. The presence of the bacterium in the gastric mucosa is associated with chronic gastritis, often accompanied by an active inflammatory component, and promotes the formation of peptic ulceration in certain infected individuals (26, 27). Retrospective seroepidemiological studies have demonstrated that individuals infected with *H. pylori* have an increased risk of developing an adenocarcinoma (29, 30). Long-term gastric colonization with *H. pylori* is thought to induce chronic atrophic gastritis (24), which is a precursor of gastric cancer (4, 24). It has therefore been proposed that eradication of the bacterium, particularly within those populations in which an *H. pylori* infection is acquired at an early age, may reduce the cases of such a neoplasm (24).

Several chemotherapeutic regimens for the treatment of *H. pylori* infection currently exist; nevertheless, the widespread treatment of individuals with antibiotics would be both unwise and impractical. Encouraging data supporting active immunization as a means of prophylaxis against *H. pylori* infection have emerged from experiments using a mouse model of gastric infection (2, 3, 7, 8).

In this model, stomachs of mice were colonized by a close relative of *H. pylori*, *Helicobacter felis* (22), a bacterial species that is autochthonous to the stomachs of cats and dogs (23). Chen et al. (2, 3) and Czinn et al. (7) showed that it was possible to protect mice from such a colonization by orogastrically immunizing animals with sonicated extracts of *H. felis*, given in combination with cholera toxin (a mucosal adjuvant). Since these early studies were reported, attention has focused on single antigens as possible candidates in an *H. pylori* vaccine; urease is one such antigen.

During the initial stages of gastric colonization, urease activity plays a role in the protection of helicobacters from luminal acidity (9, 13). Urease is a conserved trait amongst gastric *Helicobacter* spp. Moreover, *H. pylori* urease was shown to be structurally (13, 18, 34) and functionally (13) similar to that of *H. felis*. By cloning the genes encoding the ureases of *H. pylori* (20) and *H. felis* (12), we showed that these enzymes, in contrast to other microbial ureases, consist of two subunits (designated UreA and UreB) which are highly conserved at the amino acid sequence level (12). Recently, it was shown that *H. pylori* urease is a protective antigen in the *H. felis*-mouse model (8).

The aims of the study were to develop recombinant antigens derived from the urease subunits of *H. pylori* and *H. felis* and to assess the immunoprotective efficacies of these antigens in the *H. felis*-mouse model. Each of the structural genes encoding the respective urease subunits from *H. pylori* and *H. felis* was independently cloned and overexpressed in *Escherichia coli*. The resulting recombinant urease antigens (which were fused to a 42-kDa maltose-binding protein [MBP] of *E. coli*) were purified in large quantities from *E. coli* cultures and were immunogenic yet enzymatically inactive. The findings demonstrated the feasibility of developing a recombinant vaccine against *H. pylori* infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *H. felis* (ATCC 49179) was grown on a blood agar medium containing blood agar base no. 2 (Oxoid) supplemented with 10% lysed horse blood (BioMérieux) and an antibiotic supplement consisting of vancomycin (10 μg/ml), polymyxin B (25 ng/ml), trimethoprim (5 μg/ml), and amphotericin B (2.5 μg/ml).

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Bacteria were cultured under microaerobic conditions at 37°C for 2 days (12). *E. coli* MC1061 cells were grown routinely at 37°C in Luria medium. The antibiotics carbenicillin (100 μg/ml) and spectinomycin (100 μg/ml) were added as required.

DNA manipulations and analysis. All DNA manipulations and analyses, unless mentioned otherwise, were performed according to standard procedures (25). Restriction and modification enzymes were purchased from Amersham (Les Ulis, France). DNA fragments to be cloned were electroeluted from agarose gels and then purified by passage on Elutip minicolumns (Schleicher and Schüll, Dassel, Germany).

The PCR. Typical reaction samples contained 10 to 50 ng of denatured DNA; PCR buffer (50 mmol of KCl per liter in 10 mmol of Tris-HCl per liter [pH 8.3]); dATP, dGTP, dCTP, and dTTP (each at a final concentration of 1.25 mmol/liter); 2.5 mmol of $MgCl_2$ per liter; 100 pmol of each primer; and 0.5 μl of *Taq* polymerase. The samples were subjected to 30 cycles of the following program: 2 min of denaturation at 94°C, 1 min of annealing at either 40 or 55°C (depending upon the level of stringency required), and extension for 2 min at 72°C.

Cloning of amplification products in pAMP. Amplification products were cloned into the cohesive ends of the pAMP vector (Fig. 1) according to the protocol described by the manufacturer (CloneAmp System; Gibco BRL, Cergy Pontoise, France). Briefly, 60 ng of amplification product was directly mixed in a buffer (consisting of 50 mmol of KCl per liter, 1.5 mmol of $MgCl_2$ per liter, and 0.1% [wt/vol] gelatin in 10 mmol of Tris-HCl per liter, pH 8.3) with 50 ng of the pAMP 1 vector DNA and 1 U of uracil DNA glycosylase. Ligation was performed for 30 min, at 37°C. Competent cells (200 μl) of *E. coli* MC1061 were transformed with 20 μl of the ligation mixture.

Purification of recombinant urease polypeptides. The urease polypeptides from *Helicobacter* spp. were overexpressed in *E. coli* cells with the expression vector pMAL-C2 (New England Biolabs Inc., Beverly, Mass.). The pMAL-C2 vector is under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter (P_{tac}) and contains an open reading frame that encodes the synthesis of MalE (MBP). Cloning of sequences in phase with this open reading frame resulted in the production of MBP translational fusion proteins which, by virtue of the affinity between MBP and amylose, facilitated the purification of recombinant polypeptides.

Large quantities of recombinant protein were purified according to the manufacturer's instructions. Briefly, fresh 500-ml volumes of Luria broth, containing carbenicillin (100 μg/ml) and 2% (wt/vol) glucose, were inoculated with overnight cultures (5 ml) of *E. coli* clones. The cultures were incubated at 37°C and shaken at 250 rpm, until the A_{600} was 0.5. Prior to adding 1 mmol (final concentration) of IPTG per liter to cultures, a 1.0-ml sample was taken (noninduced cells). Cultures were incubated for a further 4 h, at which time another 1.0-ml sample (induced cells) was taken. The noninduced and induced cell samples were later analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

IPTG-induced cultures were spun at 7,000 rpm for 20 min in a Sorvall R-C5 centrifuge (Sorvall, Norwalk, Conn.) at 4°C. Pellets were resuspended in 50 ml of column buffer (200 mmol of NaCl per liter, 1 mmol of EDTA per liter in 10 mmol of Tris-HCl per liter [pH 7.4]), containing the following protease inhibitors (supplied by Boehringer, Mannheim, Germany): 2 μmol of leupeptin per liter, 2 μmol of pepstatin per liter, and 1 mmol of phenylmethylsulfonyl fluoride per liter. Intact cells were lysed by passage through a French pressure cell (16,000 lb/in^2). Cell debris was removed by centrifugation, and lysates were diluted in column buffer to give a final concentration of

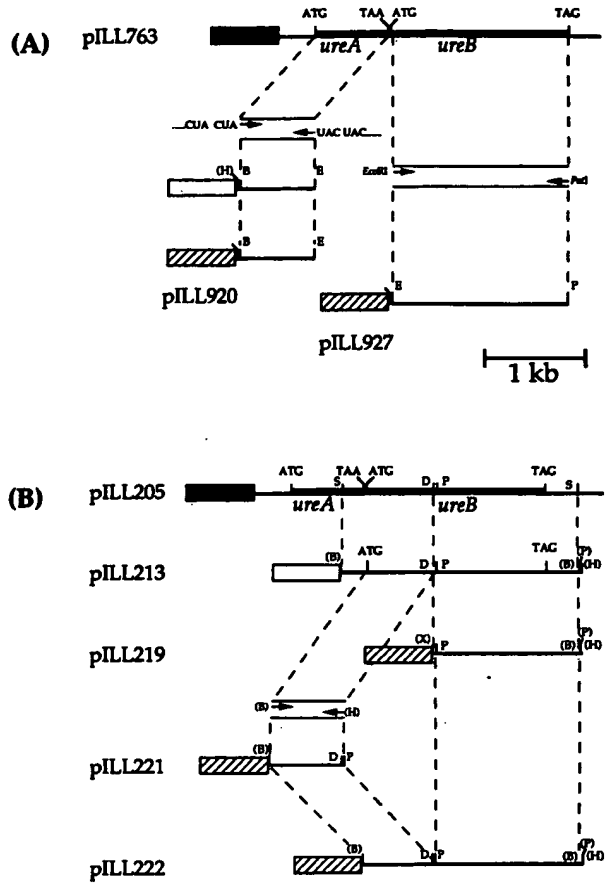

FIG. 1. Plasmid constructions for the expression of recombinant urease antigens from *H. pylori* (A) and *H. felis* (B). The respective start codons (ATG) and stop codons (TAA or TAG) of the structural genes, *ureA* and *ureB*, are indicated. Numbers refer to the plasmid constructions (Table 2). Recombinant UreA and UreB constructions for *H. pylori* (pILL920 and pILL927, respectively), as well as *H. felis* UreA (pILL919 [not shown], as per pILL920) and UreB (pILL222), were constructed by PCR as described in Materials and Methods. Arrows indicate oligonucleotide primers and include the corresponding tails introduced as cloning sites. Plasmid vectors pILL570 (blackened box), pAMP-1 (stippled box), pUC18 (empty box), and pMAL (striped box) are shown. Restriction sites are indicated by abbreviations, as follows: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sau*3A; D, *Dra*I; X, *Xmn*I. Parentheses indicate sites present on the vectors.

2.5 mg of protein per ml, prior to chromatography on a column (2.6 by 20 cm) of amylose resin (New England Biolabs). The resin was washed with column buffer at 0.5 ml/min until the A_{280} returned to baseline levels. The MBP-fused recombinant proteins were eluted from the column by washing with column buffer containing 10 mmol of *l*-maltose per liter.

Fractions containing the recombinant proteins were pooled and then dialyzed several times at 4°C against a low-salt buffer (containing 25 mmol of NaCl per liter in 20 mmol of Tris-HCl per liter [pH 8.0]). The pooled fractions were then loaded at a flow rate of 0.5 ml/min onto an anion-exchange column (1.6 by 10 cm) (HP-Sepharose; Pharmacia, Uppsala, Sweden), connected to a Hi-Load chromatography system (Pharmacia). Proteins were eluted from the column with a salt gradient (25 to 500 mmol of NaCl per liter). Fractions giving high readings

at A_{280} were exhaustively dialyzed against distilled water at 4°C and analyzed by SDS-PAGE.

Rabbit antisera. Polyclonal rabbit antisera were prepared against total cell extracts of *H. pylori* 85P (11) and *H. felis* (ATCC 49179) (12). Polyclonal rabbit antisera against recombinant protein preparations of *H. pylori* and *H. felis* urease subunits were produced by immunizing rabbits with 100 μg of purified recombinant protein in Freund's complete adjuvant. Four weeks later, rabbits were booster immunized with 100 μg of protein in Freund's incomplete adjuvant. At week 6, the animals were terminally bled and the sera were stored at −20°C.

Protein analyses by SDS-PAGE and Western blotting (immunoblotting). Solubilized cell extracts were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and a 10% resolving gel, according to the procedure of Laemmli. Electrophoresis was performed at 200 V on a mini-slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.).

Proteins were transferred to nitrocellulose paper in a Mini Trans-Blot transfer cell (Bio-Rad) set at 100 V for 1 h, with cooling. Nitrocellulose membranes were blocked with 5% (wt/vol) casein (BDH, Poole, England) in phosphate-buffered saline (PBS) with gentle shaking at room temperature, for 2 h (11). Membranes were reacted at 4°C overnight with antisera diluted in 1% casein prepared in PBS. Immunoreactants were detected with specific biotinylated secondary antibodies and streptavidin-peroxidase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Reaction products were visualized on autoradiographic film (Hyperfilm, Amersham) by a chemiluminescence technique (ECL System, Amersham).

Protein concentrations were determined by the Bradford assay (Sigma Chemical Co., St. Louis, Mo.).

Animal experimentation. Six-week-old female Swiss specific-pathogen-free mice were obtained (Centre d'Elevage R. Janvier, Le-Genest-St-Isle, France) and maintained on a commercial pellet diet with water ad libitum. To ensure that the specific-pathogen-free mice did not harbor urease-positive *Helicobacter muridarum* bacteria (13), six animals were randomly selected and the intestines and ceca of these animals were removed. The tissues were washed in saline, and mucus scrapings were then examined by phase-contrast microscopy for the presence of *H. muridarum* bacteria. The absence of *H. muridarum* from a random selection of animals suggested that the colony of mice was *H. muridarum* free. For all orogastric administrations, 100-μl aliquots were delivered to mice with 1.0-ml disposable syringes, to which polyethylene catheters (Biotrol, Paris, France) were attached.

Preparation of sonicated extracts of *H. felis*. *H. felis* bacteria were harvested in PBS and centrifuged at 5,000 rpm for 10 min in a Sorvall RC-5 centrifuge at 4°C. The pellets were washed twice and resuspended in PBS. Bacterial suspensions were sonicated as previously described (13) and were subjected to at least one freeze-thaw cycle. Protein determinations were carried out on the sonicates.

Preparation of *H. felis* inocula for immunoprotection studies. To ensure virulent cultures of *H. felis* for protection studies, bacteria were reisolated from stomach biopsies of *H. felis*-infected mice. The isolates were passaged a minimum number of times in vitro. Stock cultures of these bacteria were stored at −80°C and were used, as required, to prepare fresh inocula for subsequent mouse protection studies. This procedure ensured the reproducibility of inocula used in successive experiments. Prior to inoculation of the animals, bacterial viability and motility were assessed by phase microscopy.

Mouse protection studies. A total of 50 μg of recombinant antigen and 10 μg of cholera holotoxin (Sigma Chemical Corp.), both resuspended in HCO_3, were administered orogastrically to mice at weeks 0, 1, 2, and 3. Mice immunized with sonicated *H. felis* extracts (containing 400 to 800 μg of total protein) were also given 10 μg of cholera toxin. At week 5, half of the mice from each group were challenged with 0.1 ml of an *H. felis* inoculum (approximately 10^8 bacteria per ml). The remainder of the mice received an additional booster immunization at week 15. At week 17, the latter were challenged with 0.1 ml of *H. felis* culture (approximately 10^6 bacteria per ml).

Assessment of *H. felis* colonization of the mouse. Two weeks after receiving the challenge dose (i.e., weeks 7 and 19, respectively), mice were sacrificed by spinal dislocation. The stomachs were washed twice in sterile 0.8% NaCl, and a portion of the gastric antrum from each stomach was placed on the surfaces of agar plates (12 by 12 cm) containing a urea indicator medium (2% urea, 120 mg of Na_2HPO_4, 80 mg of KH_2PO_4, 1.2 mg of phenol red, 1.5 g of agar prepared in 100 ml). The remainder of each stomach was placed in formal-saline and stored until being processed for histology. Longitudinal sections (4 μm) of the stomachs were cut and stained by the Giemsa and hematoxylin-eosin techniques.

The presence of *H. felis* bacteria in mouse gastric mucosa was assessed by the detection of urease activity (for up to 24 h) on the indicator medium, as well as by the screening of Giemsa-stained gastric sections that had been coded so as to eliminate observer bias. Bacterial colonization was defined as the presence of any *H. felis* bacteria in gastric sections, whilst mice were considered protected when no bacteria were seen in the sections. Mononuclear cell infiltrates were scored as follows: 0, no significant infiltration; 1, infiltration of low numbers of lymphocytes, limited to the submucosa and muscularis mucosa; 2, infiltration of moderate numbers of lymphocytes to the submucosa and muscularis mucosa, sometimes forming loose aggregates; and 3, infiltration of large numbers of lymphocytes, featuring nodular agglomerations of these cells.

RESULTS

Construction of recombinant plasmids. To clone the *ureA* genes of *H. pylori* and *H. felis*, degenerate 36-mer primers were synthesized on the basis of the published urease sequences (primer set 1, Table 1). Purified DNA from *E. coli* clones harboring plasmids pILL763 and pILL205 (Table 2), which encoded the structural genes of *H. pylori* and *H. felis* ureases, was used as template material in PCRs performed under nonstringent conditions. The amplification products were inserted into the plasmid vector pAMP (Fig. 1). Inserts were subsequently excised from the polylinker of the pAMP vector by double digestion with *Bam*HI and *Pst*I and then subcloned into the expression vector pMAL, chosen for the production of recombinant antigens (pILL919 and pILL920, respectively, Fig. 1).

A product containing the *ureB* gene of *H. pylori* was amplified under stringent conditions with a set of 35-mer primers (set no. 2, Table 1). The purified amplification product (1,850 bp) was digested with *Eco*RI and *Pst*I and then cloned directly into pMAL (pILL927, Fig. 1).

H. felis ureB was cloned in a two-step procedure that allowed the production of both complete and truncated versions of the UreB subunit. Plasmid pILL213 (Fig. 1) was digested with the enzymes *Dra*I, corresponding to amino acid residue number 219 of the UreB subunit (16), and *Hin*dIII. The resulting 1,350-bp fragment was purified and cloned into pMAL that had been digested with *Xmn*I and *Hin*dIII (pILL219, Fig. 1). In order to produce a clone capable of synthesizing a complete

TABLE 1. The oligomeric primers used in PCR-based amplification of urease-encoding nucleotide sequences

Primer set	Direction[a]	Nucleotide sequence (5'→3')
1	Forw	...[b]CAU (CCN[c]) (AAR) (GAR) (YTN) (GAY) (AAR) (YTN) (ATG)
	Rev	(YTC) (YTT) (NCG) (NCG) (NSW) (DAT) (YTT) (YTT) (CAT) CUA[b]...
2	Forw	CC GGA GAA TTC ATT AGC AGA AAA GAA TAT GTT TCT ATG (*Eco*RI[d])
	Rev	AC GTT CTG CAG CTT ACG AAT AAC TTT TGT TGC TTG AGC (*Pst*I[d])
3	Forw	GGA TCC AAA AAG ATT TCA CG (*Bam*HI[d])
	Rev	GGA AGC TT C TGC AGG TGT GCT TCC CCA GTC (*Hin*dIII[d] *Pst*I[d])

[a] Forw, forward; Rev, reverse.
[b] The 5' ends of these primers each had a series of four CAU and CUA codons, respectively, that were compatible with the pAMP vector.
[c] Degenerated nucleotides were synthesized according to the following code: Y, C or T; R, A or G; S, G or C; W, A or T; D, G or A or T; and N, G or A or C or T.
[d] Restriction sites introduced in the amplified fragments.

UreB protein, PCR primers (set 3, Table 1) that amplified a 685-bp fragment from the N-terminal portion of the *ureB* gene (excluding the ATG codon), which also overlapped the beginning of the insert in plasmid pILL219, were developed. The PCR-amplified material was purified and digested with *Bam*HI and *Hin*dIII and then cloned into pMAL (pILL221, Fig. 1). A 1,350-bp *Pst*I-*Pst*I fragment encoding the remaining portion of the *ureB* gene product was subsequently excised from pILL219 and cloned into a linearized preparation of pILL221 (pILL222, Fig. 1).

Expression of *Helicobacter* urease polypeptides in *E. coli*. Recombinant urease proteins were purified from cell extracts of *E. coli* cells following chromatography on affinity (amylose resin) and anion-exchange (Q-Sepharose) gel media (Fig. 2). *E. coli* MC1061 cells transformed with recombinant plasmids encoding the respective *ureA* gene products of *H. felis* and *H. pylori* (pILL919 and pILL920, respectively) expressed fusion proteins with predicted molecular masses of approximately 68 kDa (Fig. 3). Two-liter cultures of these recombinant *E. coli* MC1061 cells typically yielded 30 mg of purified antigen.

Similarly, the large UreB subunits of *H. pylori* and *H. felis* ureases were expressed in *E. coli* (plasmids pILL927 and pILL222, respectively) and produced fusion proteins with predicted molecular masses of 103 kDa (Fig. 3). The yield in these cases was appreciably lower than that for the UreA preparations (approximately 15 mg was recovered from 2 liters of bacterial culture). Moreover, problems associated with the cleavage of the UreB polypeptides from the MBP portion of the fusion proteins were encountered (Fig. 3). These difficulties were attributed to the large sizes of the recombinant UreB polypeptides.

Western blot analyses of the UreA antigen preparations with rabbit polyclonal antisera raised against whole extracts of *H. pylori* and *H. felis* bacteria demonstrated that the antigens retained antigenicity to the homologous as well as heterologous antisera (Fig. 4). The antisera did not recognize the MBP component alone. Cross-reactivity between the urease polypeptides of *H. pylori* and *H. felis* was consistent with the high degrees of identity between the amino acid sequences of these proteins (12).

Rabbit polyclonal antisera raised against purified recombinant UreB proteins prepared from *H. pylori* and *H. felis* strongly reacted with the urease polypeptides present in recombinant UreB preparations (Fig. 5A) as well as in whole-cell extracts of the bacteria (Fig. 5B). As reported previously (12), the UreB subunit of *H. felis* urease migrated slightly higher on SDS-PAGE gels than did that of *H. pylori* (Fig. 5B).

Immunization of mice against gastric *H. felis* infection. Groups of mice were immunized four times (weeks 0 to 3) with the given antigen preparations. In a preliminary immunoprotection study, one-half of the mice from each group were challenged at week 5 with an *H. felis* inoculum containing 10^8 bacteria per ml (prepared as described in Materials and Methods).

(i) Protection at week 5. Two weeks after challenge, 85% of stomach biopsy samples from the control group of animals immunized with *H. felis* sonicate preparations were urease negative and therefore appeared to have been protected from *H. felis* infection. This compared with 20% of those from the control group of mice given MBP alone. The proportion of urease-negative stomachs for those groups of mice given the recombinant urease subunits varied from 70% (for *H. pylori*

TABLE 2. Plasmids used

Plasmid	Vector	Relevant phenotype or characteristic	Reference
pILL763	pILL570 (Sp^r)	9.5-kb fragment (*Sau*3A partial digest of *H. pylori* chromosome)	6
pILL199	pILL575 (Km^r)	35-kb fragment (*Sau*3A partial digest of *H. felis* chromosome)	12
pILL205	pILL570	11-kb fragment (*Sau*3A partial digest of pILL199)	12
pILL919	pMAL-C2 (Ap^r)	0.8-kb *Bam*HI-*Pst*I fragment containing PCR product encoding *H. felis ureA* gene	This study
pILL920	pMAL-C2	0.8-kb *Bam*HI-*Pst*I fragment containing PCR product encoding *H. pylori ureA* gene	This study
pILL927	pMAL-C2	1.8-kb *Eco*RI-*Pst*I PCR fragment encoding *H. pylori ureB* gene	This study
pILL213	pUC18 (Ap^r)	2.2-kb fragment resulting from *Sau*3A partial digest of pILL205	This study
pILL219	pMAL-C2	1.4-kb *Dra*I-*Hin*dIII fragment containing *H. felis ureB* (bp 657–1707)	This study
pILL221	pMAL-C2	0.7-kb *Bam*HI-*Pst*I PCR fragment encoding *H. felis ureB* (bp 4–667)	This study
pILL222	pMAL-C2	1.35-kb *Pst*I-*Pst*I fragment encoding *H. felis ureB* (bases 667–1707) from pILL219 cloned into linearized pILL221	This study

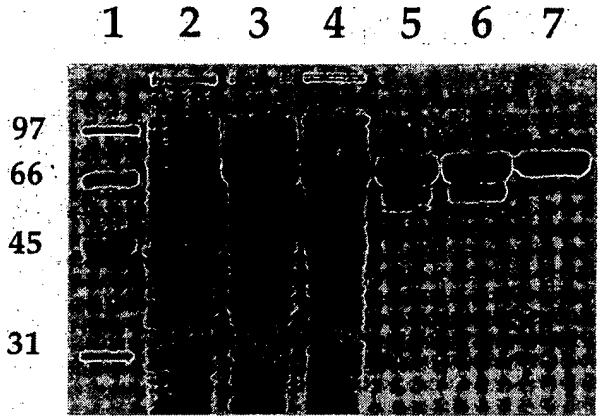

FIG. 2. Purification of *H. pylori* UreA-MBP recombinant protein by using the pMAL expression vector system. Extracts from the various stages of protein purification were electrophoresed on a resolving SDS–10% polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie blue. The extracts were as follows: lane 1, SDS-PAGE standard marker proteins (the molecular weights in thousands are shown on the side); lane 2, noninduced cells; lane 3, IPTG-induced cells; lane 4, French press lysate of induced cell extract; lane 5, eluate from amylose resin column; lane 6, eluate from anion-exchange column (first passage); and lane 7, eluate from anion-exchange column (second passage).

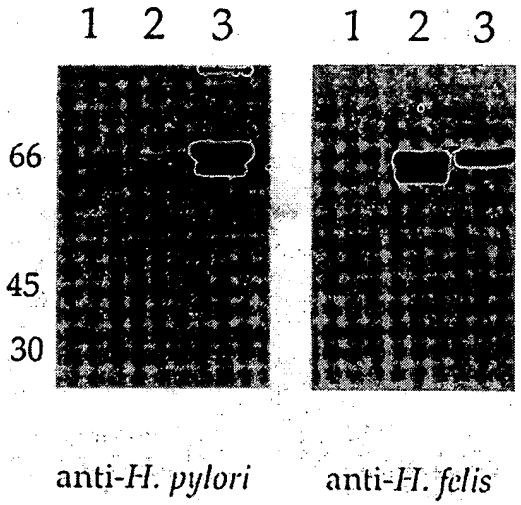

FIG. 4. Recognition of UreA recombinant fusion proteins by polyclonal rabbit anti-*Helicobacter* sp. sera. Protein extracts of MBP (lanes 1), *H. felis* UreA-MBP (lanes 2), and *H. pylori* UreA-MBP (lanes 3) were separated on a 12% polyacrylamide gel. Western blotted proteins were reacted with rabbit polyclonal antisera (diluted 1:5,000) raised against whole-cell extracts of *H. pylori* and *H. felis*. Protein degradation products reacted with the homologous rabbit sera. The numbers on the left refer to the molecular weights in thousands of standard marker proteins.

UreB) to 30% (for *H. pylori* UreA). Assessment of coded histological slides for the presence of *H. felis* bacteria, however, indicated that the levels of protection in mice were lower than that observed by the biopsy urease test: for example, only 25% of gastric tissue from the control mice immunized with *H. felis* sonicate preparations was free of *H. felis* bacteria. Gastric tissue from these mice displayed relatively fewer *H. felis* bacteria and took a relatively long time to change the color of the urease indicator medium (14). This suggested that a protective immune response had been induced in the mice but that the response was insufficient to protect against the large numbers of *H. felis* bacteria in the challenge inoculum. To test this hypothesis, mice were subsequently challenged at week 17 with an inoculum containing 100-fold fewer *H. felis* bacteria.

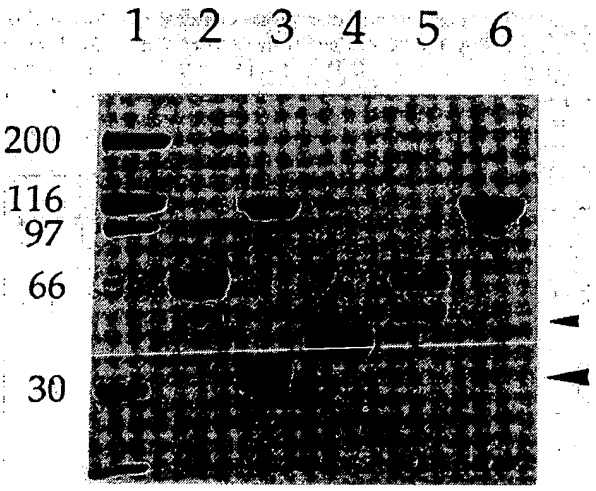

FIG. 3. Gel electrophoresis of purified recombinant urease preparations. Samples were resolved on an 8% polyacrylamide gel in the following order: lane 1, standard marker proteins; lane 2, *H. pylori* UreA-MBP; lane 3, *H. pylori* UreB-MBP; lane 4, MBP; lane 5, *H. felis* UreA-MBP; and lane 6, *H. felis* UreB-MBP. Protein degradation products (small arrowhead) and unfused MBP (large arrowhead) are indicated. The former were recognized by the homologous rabbit antiserum (Fig. 4). The numbers on the left refer to the molecular weights in thousands of standard marker proteins.

(ii) Protection at week 17. The remaining mice from each group of animals were boosted at week 15. These mice were challenged at week 17 with an *H. felis* inoculum containing 10^6 bacteria per ml. Two weeks later, all stomach biopsies from the MBP-immunized mice were urease positive (Table 3). In contrast, the proportion of mouse stomachs that were urease negative varied from 50%, for *H. pylori* UreA-immunized animals, to 100%, for those immunized with *H. felis* UreB. The latter was comparable to the level of protection observed for the group of animals immunized with *H. felis* sonicated extracts. Histological evidence demonstrated that the UreB subunits of *H. felis* and *H. pylori* protected 60 and 25% of immunized animals, respectively. For mice immunized with sonicated extracts of *H. felis*, histological analysis of tissues revealed that 85% of these animals had been protected from *H. felis* infection. Immunization of mice with recombinant *H. pylori* UreA did not protect the animals. Similarly, the stomachs of all *H. felis* UreA-immunized mice that had been challenged at week 5, and were not sacrificed until week 19, were colonized with *H. felis* bacteria (Table 3).

The urease gastric biopsy test, compared with histological analysis of gastric tissue sections, gave sensitivity and specificity values of 63 and 95%, respectively. Thus, histology proved to be the more accurate predictor of *H. felis* infection in the mouse.

Cellular immune response in immunized stomachs. In addition to the histological assessment of *H. felis* colonization,

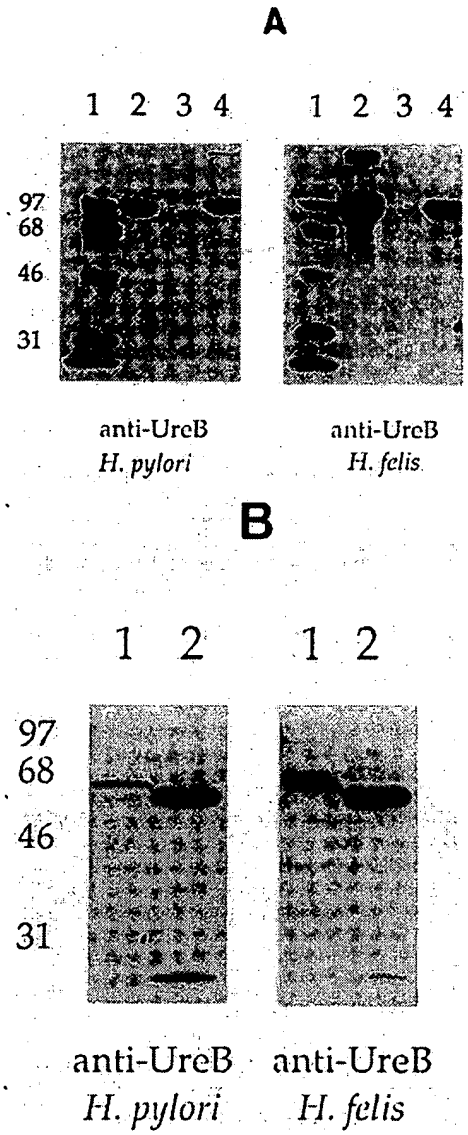

FIG. 5. Western blotting analyses with antisera raised against purified *H. pylori* and *H. felis* UreB recombinant proteins. (A) Nitrocellulose membranes were immunoblotted with antisera raised against the following purified recombinant protein extracts: lanes 1, biotinylated standard protein markers; lanes 2, *H. felis* UreB-MBP; lanes 3, MBP; and lanes 4, *H. pylori* UreB-MBP. (B) Recognition of UreB polypeptides in whole-cell extracts of *H. felis* (lanes 1) and *H. pylori* (lanes 2). Rabbit antiserum was diluted 1:5,000. The numbers on the left refer to the molecular weights in thousands of standard marker proteins.

mouse gastric tissue was scored (from 0 to 3) for the presence of lymphocytic infiltrates (Fig. 6). In mice immunized with MBP alone, a mild chronic gastritis was seen with small numbers of lymphocytes restricted to the muscularis mucosa and to the submucosa of the gastric epithelium. In contrast, there were considerable numbers of mononuclear cells present in the gastric mucosae from animals immunized with either the recombinant urease polypeptides or *H. felis* sonicate preparations (Fig. 6 and 7A). These inflammatory cells sometimes

TABLE 3. Protection of mice from *H. felis* infection following immunization with recombinant urease proteins

Antigen	No. of mice colonized by *H. felis*[a]	
	Urease[b]	Histology[c]
MBP	100 (10/10)	100 (10/10)
UreA *H. pylori*	50 (4/8)	100 (8/8)
UreA *H. felis*[d]	87.5 (7/8)	100 (8/8)
UreB *H. pylori*	35 (3/8)	75 (6/8)
UreB *H. felis*	0 (0/7)	40 (2/7)
H. felis sonicate	0 (0/8)	15 (1/8)

[a] Unless stated otherwise, mice had been immunized weekly (weeks 0 to 3), booster immunized at week 15, challenged with 10^5 *H. felis* bacteria per mouse at week 17, and sacrificed at week 19.

[b] The percentage of stomachs giving a positive urease biopsy test. Total numbers of mice are given in the parentheses.

[c] The percentage of stomachs with *H. felis* bacteria identified in histological sections of mouse gastric mucosa. Total numbers of mice are given in the parentheses.

[d] Mice had been immunized weekly (weeks 0 to 3), challenged at week 5 (with 10^7 bacteria), and sacrificed at week 19.

coalesced to form lymphoid nodules that extended into the submucosal regions of the gastric epithelia (Fig. 7B and C). The mononuclear cell response appeared to be independent of the presence of bacteria.

DISCUSSION

Individuals infected with *H. pylori* produce vast quantities of specific immunoglobulin G (IgG) antibodies in the serum (1, 31, 32), as well as IgA and IgG antibodies in the mucosal tissue (32). Despite the strong immune response, *H. pylori* bacteria remain firmly entrenched in the gastric mucosa. Consequently, immunization was for a long time dismissed as a method of prophylaxis against *H. pylori* infection. Impetus for the development of an anti-*H. pylori* vaccine, nevertheless, came from the results of several studies demonstrating the induction of protective mucosal immune responses against *H. felis* infection in mice. In the initial studies, sonicated *H. felis* extracts were used as the antigen (2, 3, 7); more recently, it was shown that *H. pylori* urease, purified either from the organism itself or

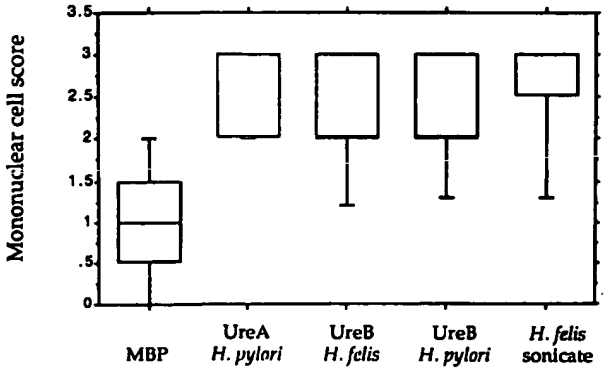

FIG. 6. Box plot representation of the distribution of mononuclear cell scores for the different immunization groups of mice. Mice had been immunized once per week (weeks 0 to 3), booster immunized at week 15, and challenged with an *H. felis* culture at week 17. Two weeks postchallenge, the mice were sacrificed. For each box plot figure, the highest point represents the 90th percentile while the lowest point represents the 10th percentile.

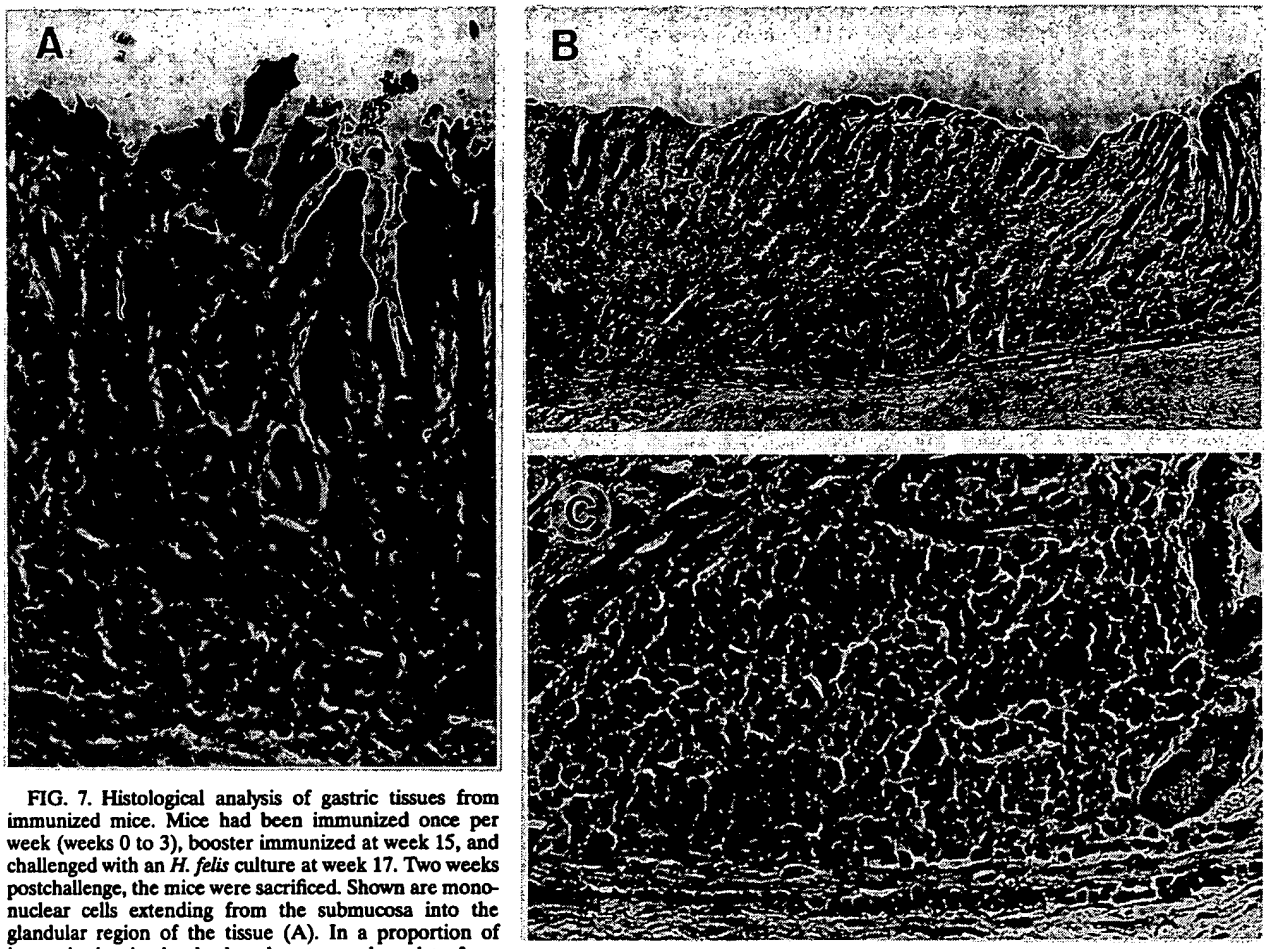

FIG. 7. Histological analysis of gastric tissues from immunized mice. Mice had been immunized once per week (weeks 0 to 3), booster immunized at week 15, and challenged with an *H. felis* culture at week 17. Two weeks postchallenge, the mice were sacrificed. Shown are mononuclear cells extending from the submucosa into the glandular region of the tissue (A). In a proportion of immunized animals, the lymphocytes coalesced to form lymphoid follicles in the subglandular region (B and C). Hematoxylin and eosin stain.

from recombinant *E. coli* cells, conferred protective immunity in mice (8). In order to determine whether this immunity might be conferred by one or more domains of the urease holoenzyme, recombinant urease subunit antigens from *H. pylori* and *H. felis* were expressed and characterized. An important aspect of the study was to compare the performance of heterologous and homologous *Helicobacter* antigens as mucosal immunogens in the *H. felis*-mouse model. The results from these studies allow us to propose the large urease subunit (UreB) as a potential component of a future *H. pylori* vaccine.

The respective UreA and UreB subunits of *H. pylori* and *H. felis* ureases were overexpressed in *E. coli* cells and purified as MBP-fused proteins. Western blot analyses using anti-*Helicobacter* rabbit sera indicated that the urease recombinant proteins were strongly immunogenic (Fig. 4 and 5). Moreover, *H. pylori* UreA and UreB recombinant proteins were recognized by sera from patients with confirmed cases of *H. pylori* disease (33). Purified MBP alone did not appear to cross-react with the rabbit antisera and so did not contribute significantly to the immunogenicity of the fusion proteins. In agreement with previous biochemical (13, 18, 34) and molecular (12) studies, immunological cross-reactivity between the recombinant urease subunits of *H. pylori* and *H. felis* was found. The cross-reactivity appeared to be greatest when anti-*H. felis* sera were used in Western blot analyses (Fig. 4 and 5B).

Though the UreB subunits of *H. felis* and *H. pylori* share an important number of immunogenic epitopes, the recombinant antigens derived from these proteins seemed to protect mice from gastric helicobacter infection to varying degrees. Hence, it is unlikely that a *Helicobacter* sp.-specific urease epitope might be sufficient to serve as a protective antigen in a vaccine. Given that heterologous *H. pylori* urease holoenzyme protected mice from gastric *H. felis* colonization (8), one might postulate that UreA, though not protective per se, may nonetheless be important in the presentation of immunoprotective domains.

The *H. felis* inoculum used in the challenge procedure was found to be an important variable in immunoprotection studies. Amongst the different mouse protection trials, the bacterial densities of the *H. felis* inocula, as well as the methods of preparing the inocula, have varied greatly. This may, in part, account for the different levels of protection (varying from 35 to 85% protection) reported by the various studies (2, 3, 7, 8). By maintaining virulent cultures of *H. felis*, the quantity of bacteria needed to colonize the mouse was significantly reduced. Using this method, we have been able to colonize mice

with as little as 10^3 *H. felis* bacteria (14). This approach should ensure reproducibility between different immunoprotection trials.

There have been several studies of the immune responses induced in the gastric mucosa of persons infected with *H. pylori* (5, 10, 19) and in animals experimentally infected with *Helicobacter* spp. (15–17, 22). In their original description of the *H. felis*-mouse model, Lee and colleagues (22) reported that, within the first 2 weeks of infection, *H. felis*-infected mice developed an acute inflammation composed predominantly of eosinophils and neutrophils. Moreover, lymphocytes did not become a predominating cell type until 8 weeks postinfection (22). The effect of mucosal immunization on gastric pathology has, thus far, not been investigated.

In this study, pronounced lymphocytic infiltrations were observed in mice that had been immunized with either *Helicobacter* urease antigens or *H. felis* sonicated extracts (Fig. 6 and 7). A particularly interesting finding was the presence of follicular structures, resembling gut-associated lymphoid tissue, in the gastric mucosa of the immunized mice. Such structures were previously described for mice that had been experimentally infected for over 1 year with *H. felis* bacteria (15, 21). Though it may be argued that the lymphocytic gastritis seen in the immunized mice was induced in response to the bacterial challenge, this seems unlikely. As reported above, lymphocyte numbers appear to increase only during the chronic stages of the murine infection (15, 21, 22), and thus the 2-week postchallenge period would have been insufficient for the development of a bacterial gastritis. Furthermore, the MBP-immunized mice, which were all colonized with *H. felis*, had relatively low infiltration scores (Fig. 6).

Mucosal immune responses normally require the uptake and presentation of antigens to lymphocytes at so-called inductor sites (28). The stimulated lymphocytes undergo differentiation and migrate to the given effector site, where specific IgA-secreting B cells proliferate and produce protective antibodies against the infectious agent (28). It is possible to speculate that the infiltrating lymphocytic cells seen in the stomachs of immunized mice may be involved in either antigen uptake or secretory IgA production. Further studies are required to address these questions as well as the types of clonal populations composing these mononuclear infiltrations.

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REFERENCES

1. **Booth, L., G. Holdstock, H. MacBride, P. Hawtin, J. R. Gibson, A. Ireland, J. Bamforth, C. E. DuBoulay, R. S. Lloyd, and A. D. Pearson.** 1986. Clinical importance of *Campylobacter pyloridis* and associated serum IgG and IgA antibody responses in patients undergoing upper gastrointestinal endoscopy. J. Clin. Pathol. **39:** 215–219.
2. **Chen, M., A. Lee, and S. L. Hazell.** 1992. Immunisation against gastric helicobacter infection in a mouse/*Helicobacter felis* model. Lancet **339:**1120–1121.
3. **Chen, M., A. Lee, S. L. Hazell, P. Hu, and Y. Li.** 1993. Immunisation against gastric infection with *Helicobacter* species: first step in the prophylaxis of gastric cancer? Zentralbl. Bakteriol. **280:**155–165.
4. **Correa, P.** 1988. A human model of gastric carcinogenesis. Cancer Res. **48:**3554–3560.
5. **Crabtree, J. E., T. M. Shallcross, R. V. Heatley, and J. I. Wyatt.** 1991. Mucosal tumour necrosis factor alpha and interleukin 6 in patients with *Helicobacter pylori*-associated gastritis. Gut **32:**1473–1477.
6. **Cussac, V., R. L. Ferrero, and A. Labigne.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J. Bacteriol. **174:**2466–2473.
7. **Czinn, S., A. Cai, and J. Nedrud.** 1993. Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. Vaccine **11:**637–642.
8. **Davin, C., A. L. Blum, I. Corthesy-Theulaz, E. Saraga, J.-P. Kraehenbuhl, R. Haas, and P. Michetti.** 1993. *H. pylori* urease elicits protection against *H. felis* infection in mice, abstr. 1213, p. A-304. Abstr. Am. Gastroenterol. Assoc. Meet. 1993.
9. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect. Immun. **62:**3604–3607.
10. **Engstrand, L., A. Scheynius, C. Pahlson, L. Grimelius, A. Schwan, and S. Gustavsson.** 1989. Association of *Campylobacter pylori* with induced expression of class II transplantation antigens on gastric epithelial cells. Infect. Immun. **57:**827–832.
11. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174:**4212–4217.
12. **Ferrero, R. L., and A. Labigne.** 1993. Cloning, expression and sequencing of *Helicobacter felis* urease genes. Mol. Microbiol. **9:** 323–333.
13. **Ferrero, R. L., and A. Lee.** 1991. The importance of urease in acid protection for the gastric-colonising bacteria *Helicobacter pylori* and *Helicobacter felis* sp. nov. Microb. Ecol. Health Dis. **4:**121–134.
14. **Ferrero, R. L., J.-M. Thiberge, and A. Labigne.** Unpublished data.
15. **Fox, J. G., M. Blanco, J. C. Murphy, N. S. Taylor, A. Lee, Z. Kabok, and J. Pappo.** 1993. Local and systemic immune responses in murine *Helicobacter felis* active chronic gastritis. Infect. Immun. **61:**2309–2315.
16. **Fox, J. G., P. Correa, N. S. Taylor, A. Lee, G. Otto, J. C. Murphy, and R. Rose.** 1990. *Helicobacter mustelae*-associated gastritis in ferrets. An animal model of *Helicobacter pylori* gastritis in humans. Gastroenterology **99:**352–361.
17. **Fox, J. G., A. Lee, G. Otto, N. S. Taylor, and J. C. Murphy.** 1991. *Helicobacter felis* gastritis in gnotobiotic rats: an animal model of *Helicobacter pylori* gastritis. Infect. Immun. **59:**785–791.
18. **Gootz, T. D., G. I. Perez-Perez, J. Clancy, B.-A. Martin, A. Tait-Kamradt, and M. J. Blaser.** 1994. Immunological and molecular characterization of *Helicobacter felis* urease. Infect. Immun. **62:**793–798.
19. **Karttunen, R.** 1991. Blood lymphocyte proliferation, cytokine secretion and appearance of T cells with activation surface markers in cultures with *Helicobacter pylori*. Comparison of the responses of subjects with and without antibodies to *H. pylori*. Clin. Exp. Immunol. **83:**396–400.
20. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173:**1920–1931.
21. **Lee, A., M. Chen, N. Coltro, J. O'Rourke, S. L. Hazell, P. Hu, and Y. Li.** 1993. Long term infection of the gastric mucosa with *Helicobacter* species does induce atrophic gastritis in an animal model of *Helicobacter pylori* infection. Zentralbl. Bakteriol. **280:** 38–50.
22. **Lee, A., J. G. Fox, G. Otto, and J. Murphy.** 1990. A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology **99:**1315–1325.
23. **Lee, A., S. L. Hazell, J. O'Rourke, and S. Kouprach.** 1988. Isolation of a spiral-shaped bacterium from the cat stomach. Infect. Immun. **56:**2843–2850.
24. **Leon-Barua, R., S. Recavarren-Arce, R. H. Gilman, and R. Berendson.** 1993. Can eradication of *Helicobacter pylori* prevent gastric cancer? Drugs **46:**341–346.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, N.Y.
26. **Marshall, B. J., C. S. Goodwin, J. R. Warren, R. Murray, E. D. Blincow, S. J. Blackbourn, M. Phillips, T. E. Waters, and C. R. Sanderson.** 1988. Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. Lancet ii:1439–1442.
27. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet i:1311–1315.
28. **McGhee, J. R., and H. Kiyono.** 1993. New perspectives in vaccine development: mucosal immunity to infections. Infect. Agents Dis. 2:55–73.
29. **Nomura, A., G. N. Stemmermann, P.-H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. 325:1132–1136.
30. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. 325:1127–1131.
31. **Perez-Perez, G. I., B. Dworkin, J. Chodos, and M. J. Blaser.** 1988. *Campylobacter pylori*-specific serum antibodies in humans. Ann. Intern. Med. 109:11–17.
32. **Rathbone, B. J., J. I. Wyatt, B. W. Worsley, S. E. Shires, L. K. Trejdosiewicz, R. V. Heatley, and M. S. Losowsky.** 1986. Systemic and local antibody responses to gastric *Campylobacter pyloridis* in non-ulcer dyspepsia. Gut 27:642–647.
33. **Thiberge, J.-M., R. L. Ferrero, and A. Labigne.** Unpublished data.
34. **Turbett, G. R., P. B. Hoj, R. Horne, and B. J. Mee.** 1992. Purification and characterization of the urease enzymes of *Helicobacter* species from humans and animals. Infect. Immun. 60: 5259–5266.

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Effect of Oral Immunization with Recombinant Urease on Murine *Helicobacter felis* Gastritis

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The ability of oral immunization to interfere with the establishment of infection with *Helicobacter felis* was examined. Groups of Swiss Webster mice were immunized orally with 250 μg of *Helicobacter pylori* recombinant urease (rUrease) and 10 μg of cholera toxin (CT) adjuvant, 1 mg of *H. felis* sonicate antigens and CT, or phosphate-buffered saline (PBS) and CT. Oral immunization with rUrease resulted in markedly elevated serum immunoglobulin G (IgG), serum IgA, and intestinal IgA antibody responses. Challenge with live *H. felis* further stimulated the urease-specific intestinal IgA and serum IgG and IgA antibody levels in mice previously immunized with rUrease but activated primarily the serum IgG compartment of PBS-treated and *H. felis*-immunized mice. Intestinal IgA and serum IgG and IgA anti-urease antibody responses were highest in rUrease-immunized mice at the termination of the experiment. Mice immunized with rUrease were significantly protected ($P \leq 0.0476$) against infection when challenged with *H. felis* 2 or 6 weeks post-oral immunization in comparison with PBS-treated mice. Whereas *H. felis*-infected mice displayed multifocal gastric mucosal lymphoid follicles consisting of CD45R$^+$ B cells surrounded by clusters of Thy1.2$^+$ T cells, gastric tissue from rUrease-immunized mice contained few CD45R$^+$ B cells and infrequent mucosal follicles. These observations show that oral immunization with rUrease confers protection against *H. felis* infection and suggest that gastric tissue may function as an effector organ of the mucosal immune system which reflects the extent of local antigenic stimulation.

Infection with *Helicobacter pylori* is associated with the development of gastritis, peptic ulceration, and gastric carcinoma (1, 27). Gastric tissues infected with *Helicobacter* spp. harbor lymphoid follicles (12, 32) driven by local antigen stimulation (40). Populations of CD3$^+$, CD4$^+$, and αβ T-cell receptor (αβTCR)-positive T cells recruited into gastric mucosa may regulate B-cell function and local immunoglobulin A (IgA) antibody secretion (12, 35). While *H. pylori* antigens activate peripheral blood T cells and B cells in vitro (20), the antigen specificity of lymphocytes resident in gastric mucosae has not been examined directly. Serum IgG and local mucosal IgA antibody responses are developed in response to infection with *Helicobacter* spp., although these appear to be insufficient for clearance (5, 12, 30, 37).

The urease molecule from *H. pylori* is an essential determinant of pathogenicity. Because urease-negative mutants fail to colonize gastric tissue of gnotobiotic piglets (8) and nude mice (36), urease may represent an important target for immunization and prevention of disease. Urease is a multimeric enzyme composed of two structural subunits of 29.5 (UreA) and 66 (UreB) kDa (7, 18) and is localized in both the cytoplasm and on the surface of *H. pylori* (15). The *H. pylori* urease molecule exhibits a high degree of sequence conservation in comparison with that of *Helicobacter felis* urease (11).

A model of chronic persistent gastritis using mice infected with *H. felis* has allowed the study of immune responses and disease progression in chronically infected animals (12, 23). Using this model, recent studies have shown that oral immunization with *H. felis* antigens results in protection against subsequent challenge with *H. felis* organisms (2, 6, 22). While mucosal immunization strategies which prevent *Helicobacter* infection generate mucosal IgA and serum IgG and IgA antibodies (2, 6), parenteral immunization yielding high levels of serum IgG does not confer protection against challenge (3, 9). The experiments presented herein examined the ability of orally administered recombinant urease (rUrease) antigen to protect mice from *H. felis* infection and *H. felis*-associated gastritis. We show the mucosal IgA and serum IgG and IgA antibody responses in immunized mice subsequently challenged with live *H. felis* and the gastric lymphocyte cytoarchitecture in infected and protected mice.

MATERIALS AND METHODS

Animals. Twenty-eight germfree 4-week-old female Swiss Webster mice were obtained from Taconic Farms (Germantown, N.Y.). The mice were maintained in a germfree isolator and subsequently housed in barrier conditions (12) for the duration of the experimental treatments. All materials for the germfree unit were sterilized by peracetic acid, and the mice were fed an autoclaved pelleted diet and given sterile water ad libitum.

Bacteria. *H. pylori* ATCC 43505 was cultured on Mueller-Hinton agar plates supplemented with 5% defibrinated sheep blood and 10 μg of vancomycin per ml, 10 μg of trimethoprim per ml, and 10 μg of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) per ml. *Escherichia coli* BL21-DE3 (Novagen, Madison, Wis.) harboring plasmid pORV154 was cultured in liquid medium in Luria broth (Difco Laboratories, Detroit, Mich.) containing 50 μg of ampicillin (Sigma) per ml or on Luria broth plates containing 1.5% agar and 100 μg of ampicillin per ml. The *H. felis* ATCC 49179 used for oral challenge was grown under microaerobic conditions on 5% lysed horse blood agar supplemented with 10 μg of vancomycin per ml, 5 μg of trimethoprim lactate per ml, 3 μg of polymyxin B (Sigma) per ml, and 2.5 μg of amphotericin per ml as described elsewhere (23). The bacteria were harvested, inoculated in brain heart infusion agar with 30% glycerol, and frozen at −70°C. Prior to use, aliquots were thawed, analyzed for motility, and cultured for evidence of aerobic or anaerobic bacterial contamination.

Preparation of *H. felis* antigens and native *H. pylori* urease. *H. felis* sonicate antigens were prepared as described elsewhere (13). In brief, *H. felis* was grown for 48 h in brucella broth (Difco) containing 5% fetal calf serum. Cultures were incubated at 37°C in a microaerobic environment and shaken at 120 rpm. The cultures were centrifuged at 10,000 rpm (Sorvall RC-5B, Newtown, Conn.) for 10 min, the pellet was washed in phosphate-buffered saline (PBS), and the cells were disrupted by sonication (Artec K System, Inc., Farmingdale, N.Y.). After

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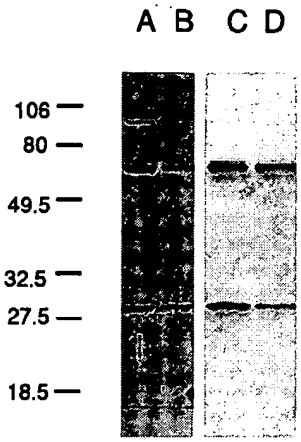

FIG. 1. Characterization of *H. pylori* native urease and rUrease. Purified urease (5 μg) derived from *E. coli* containing the UreA and UreB genes of *H. pylori* (lanes A and C) or from *H. pylori* ATCC 43505 (lanes B and D) was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels (lanes A and B) or transferred to nitrocellulose for Western blotting (lanes C and D) with mouse polyclonal antibody raised against *H. pylori* apoenzyme. Molecular size standards are shown in kilodaltons.

centrifugation at 10,000 rpm (Sorvall RC-5B; Dupont) for 10 min, the protein content was determined (25) and aliquots were frozen at −70°C until used. Urease from *H. pylori* was purified from organisms harvested from blood agar plates. The *H. pylori* organisms were lysed, centrifuged, and subjected to chromatography on DEAE-Sepharose (Pharmacia, Piscataway, N.J.) (18). The bound material was eluted with 150 mM NaCl, concentrated, and applied to a Sephacryl 300 sizing column (Pharmacia). Eluted fractions were assayed for the presence of urease activity (16). Fractions containing urease activity were then bound to Mono-Q Sepharose (Pharmacia) and eluted with a 0 to 1 M NaCl gradient. The fractions containing urease activity were pooled, concentrated, and stored in 50% glycerol at −20°C.

Cloning and purification of *H. pylori* rUrease. *H. pylori* rUrease was derived from *E. coli* ORV154 expressing the structural genes for the A (UreA) and B (UreB) subunits (4, 21) required for assembly (17). ORV154 was constructed as described elsewhere (24). rUrease was expressed constitutively from the T7 promoter (33) of pGEM3Z and purified as follows. ORV154 was cultured overnight at 37°C in shake flasks. The organisms were harvested by centrifugation, washed in PBS, and lysed by sonication. Contaminating proteins were removed from cell extracts by chromatography on DEAE-Sepharose (Pharmacia) and then by a 50 mM NaCl wash. The eluate was diluted 10- to 20-fold and then bound to DEAE-Sepharose. Urease was eluted with 150 mM NaCl, and fractions containing urease were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) (34) using a mouse polyclonal antibody raised against *H. pylori* urease. The urease-containing fractions were pooled, concentrated, and subjected to Sephacryl 300 sizing chromatography. This procedure yielded enzymatically inactive rUrease exhibiting two major bands of 60 and 30 kDa, corresponding to the B and A subunits, respectively (Fig. 1), with >90% purity, as assessed by scanning densitometry (Pharmacia-LKB UltroScan).

Experimental protocol for oral immunization. Mice were divided into three groups and immunized per os on days 0, 10, 20 and 30 with a blunt feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) as follows: group 1, PBS and 10 μg of cholera toxin (CT; Calbiochem, La Jolla, Calif.; $n = 8$); group 2, 1 mg of *H. felis* sonicate and 10 μg of CT ($n = 10$); and group 3, 250 μg of *H. pylori* rUrease plus 10 μg of CT ($n = 10$). Groups of mice were challenged with live *H. felis* three times at 2-day intervals (12) either 2 weeks (day 45) or 6 weeks (day 73) after the last oral immunization.

Culture for *H. felis* and urease tests. From 31 to 32 days (2-week postimmunization groups) or 41 to 42 days (6-week postimmunization groups) post-*H. felis* challenge, the mice were euthanized with an overdose of carbon dioxide. Two-millimeter cubes of gastric mucosa from the antrum, fundus, and duodenum were collected aseptically for culture or for the tissue urease test (12, 16).

Determination of antibody levels to urease and *H. felis* antigens in serum and feces. Blood was obtained from the retro-orbital sinus 7 days after the second and fourth immunizations, 13 to 14 days after *H. felis* challenge, and by cardiac puncture at the termination of the experiment. Secretory IgA was extracted from fecal pellets (14) by incubation in PBS containing 5% nonfat dry milk, 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (Calbiochem), 1 μg of aprotinin per ml, 10 μM leupeptin (Sigma), and 3.25 μM bestatin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). After extensive vortexing, the fecal material was centrifuged (16,000 × *g* for 10 min), and the supernatants were used for determination of antibody. An enzyme-linked immunosorbent assay (ELISA) was used for antibody measurement. In brief, triplicate wells of microtiter plates (Dynatech, Chantilly, Va.) were incubated with purified *H. pylori* urease or with *H. felis* sonicate preparations (100 μg/ml) in carbonate buffer. After washing with PBS–0.5% Tween 20, the wells were blocked with PBS-Tween containing 2.5% nonfat dry milk and incubated for 1 h at 37°C with serial dilutions of sera or fecal extracts. The wells were then incubated with biotinylated goat-anti mouse IgG or goat-anti-mouse IgA (Southern Biotechnology, Birmingham, Ala.) and then with streptavidin-alkaline phosphatase (Calbiochem). Negative control sera and fecal extracts and positive serum controls with known anti-*H. felis* activity were included in each assay.

TABLE 1. Effect of oral immunization on the presence of *H. felis* in gastric tissue[a]

Treatment group	Time postimmunization of *H. felis* challenge (wk)	No. of mice positive/total no. by:		
		Urease test	Culture	Warthin-Starry histology
PBS	2	3/4	4/4	3/4
H. felis sonicate	2	0/5[b]	1/5[c]	0/5[b]
H. pylori rUrease	2	0/5[b]	0/5[d]	0/5[b]
PBS	6	1/4	3/4	2/4
H. felis sonicate	6	0/5	0/5[b]	0/5
H. pylori rUrease	6	0/5	0/5[b]	0/5

[a] Groups of germfree Swiss Webster mice were orally immunized and challenged per os three times at 2-day intervals with 10^8 *H. felis* organisms. After 31 to 32 days (2-week postimmunization groups) or 41 to 42 days (6-week postimmunization groups), gastric tissue was examined for the presence or absence of *H. felis* infection as indicated.

[b] $P = 0.0476$ by Fisher's exact test compared with value of corresponding PBS group.

[c] $P = 0.0238$ by Fisher's exact test compared with value of corresponding PBS group.

[d] $P = 0.0079$ by Fisher's exact test compared with value of corresponding PBS group.

Histopathology. Longitudinal sections of gastric tissue, from the esophageal junction through the duodenum, were fixed in 10% neutral buffered formalin. Stomachs were processed for routine histology and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Sections of the fundus, antrum, and duodenal-pyloric junction were examined in a coded fashion for histological changes and for the presence of *H. felis* in Warthin-Starry-stained specimens.

Immunohistochemistry. Longitudinal sections of gastric tissue including the corpus and antrum were mounted in O.C.T. compound (Miles Scientific, Naperville, Ill.) and frozen in liquid-nitrogen-cooled Freon 22 (12). Tissue sections (7 μm) were fixed with acetone, and biotin-avidin-binding sites were blocked for 30 min (Vector Laboratories, Burlingame, Calif.). Tissue sections were incubated with biotinylated monoclonal antibodies (MAb; see below) and then with avidin conjugated to biotinylated horseradish peroxidase (ABC; Vector Laboratories) as described elsewhere (12). Controls included incubation with a MAb of unrelated specificity. Cell-bound peroxidase was visualized with 0.05% diaminobenzidine tetrahydrochloride (Organon Teknika, Durham, N.C.) and 0.01% H_2O_2 in PBS, and sections were counterstained with methyl green. Baseline values of infiltrating leukocytes were established with groups of known PBS-treated and challenged mice. The tissue sections from the remaining experimental groups for immunohistology were scored by a code. The degree of gastric infiltration and/or expression of antigens defined by the MAb was scored as mild (+), moderate (++), or severe (+++).

MAb. The following MAb recognizing lymphocyte cell surface structures were used in this study: anti-Thy1.2 (clone 30-H12), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7) (Becton Dickinson, San Jose, Calif.), anti-CD3 (clone 145-2C11), anti-CD5 (clone 53-7.3), anti-CD49d (VLA-4; clone MFR 4.B), anti-CD62L (L-selectin; clone MEL-14), anti-αβTCR (clone H57-597), anti-γδTCR (clone GL3), anti-I-A^d (clone AMS-32.1), and anti-I-A^p (clone 7-16.17) (Pharmingen, San Diego, Calif.). The MAb anti-CD45R (B220; clone RA3-3A1/6.1), anti-IgM (clone 331.12), and anti-CD11b (M1/70.15) were obtained from the American Type Culture Collection (Rockville, Md.). The MAb 10-4.22 was used to identify IgA-positive B cells (13, 31).

RESULTS

Effect of oral immunization on *H. felis* infection. The outcome of gastric urease tests, bacterial cultures, and histological identification of *H. felis* organisms by Warthin-Starry stain of

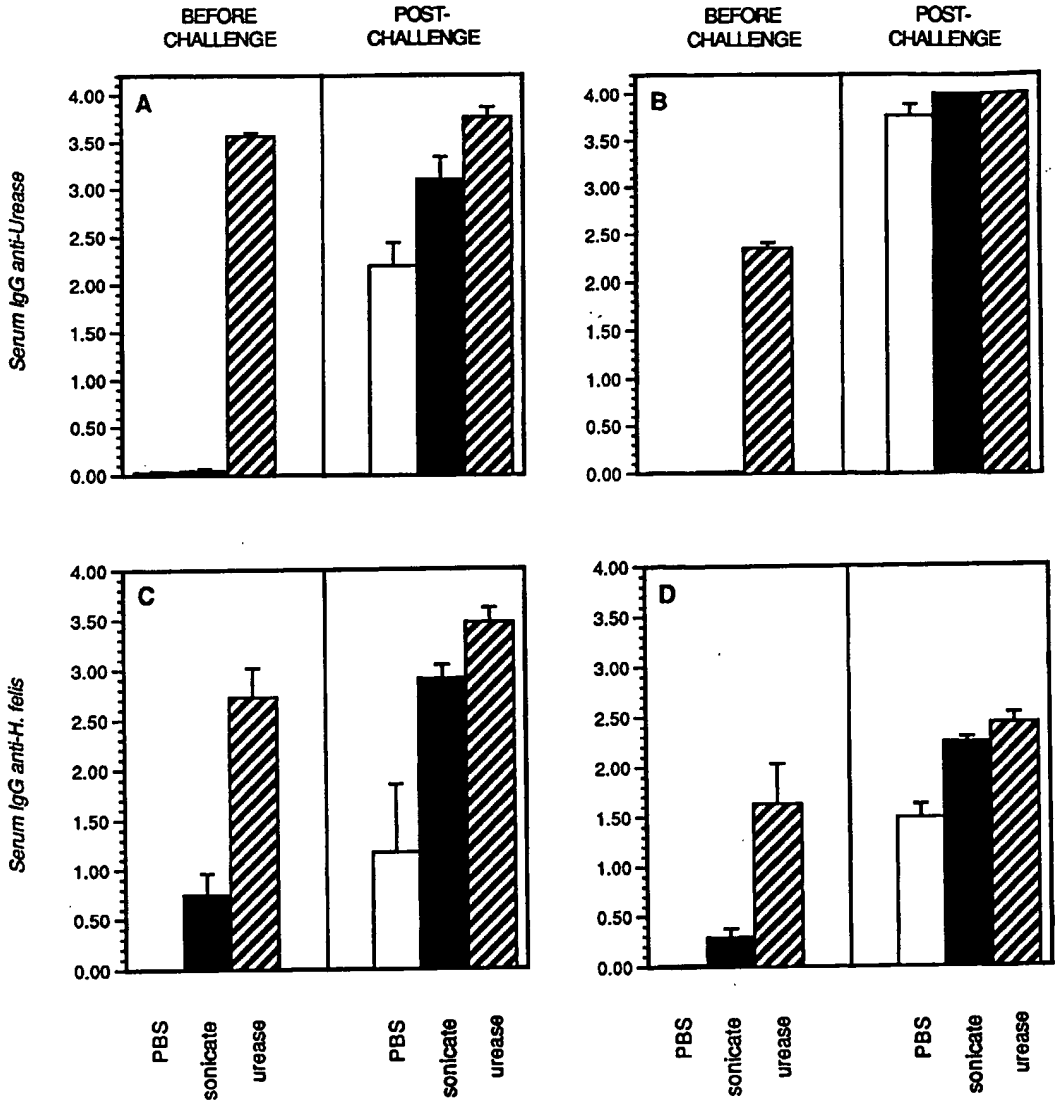

FIG. 2. Antibody responses in sera of immunized mice. Groups of Swiss Webster mice were immunized orally with PBS and 10 μg of CT (open bars; $n = 4$), 1 mg of *H. felis* sonicate and 10 μg of CT (filled bars; $n = 5$), or 250 μg of *H. pylori* rUrease and 10 μg of CT (striped bars; $n = 5$). The mice were challenged with live *H. felis* 2 weeks (A and C) or 6 weeks (B and D) later, and serum IgG anti-urease antibody (A and B) or serum IgG anti-*H. felis* antibody (C and D) levels were quantitated by ELISA. Values from immunized animals before challenge with *H. felis* were derived from samples taken 7 days after the last immunization. Values from postchallenge mice were obtained by analysis of samples harvested 13 to 14 days after *H. felis* challenge. The bars show the mean optical density at 405 nm for each group, and the brackets enclose 1 standard error of the mean.

gastric tissue is shown in Table 1. Immunization with *H. pylori* rUrease or with *H. felis* sonicate antigens interfered ($P \leq 0.0476$) with the establishment of infection upon challenge with live *H. felis* 2 or 6 weeks after the last immunization dose. The gastric tissues of PBS-treated mice challenged with *H. felis* remained colonized during the time periods examined.

Serum antibody levels to *H. pylori* urease and *H. felis* antigens in orally immunized mice. Oral immunization with rUrease resulted in the development of urease-specific serum IgG in 9 of 10 mice after two immunizations and in marked elevation of serum IgG anti-urease antibody in 10 of 10 mice following four immunizations (Fig. 2A and B). Serum IgA antibody was assayed consistently in rUrease-immunized mice only after the fourth oral immunization. Specific antibody responses directed against urease were not measurable in PBS-treated mice nor in mice immunized with *H. felis* sonicate. Oral immunization with rUrease also elicited a greater serum IgG antibody against *H. felis* sonicate antigens than that generated in mice immunized with *H. felis* sonicate (Fig. 2C and D). Challenge of mice with *H. felis* either 2 or 6 weeks postimmunization further stimulated the serum IgG anti-urease antibody response of rUrease-immunized mice and activated the serum IgG antibody compartment of PBS-treated and *H. felis*-immunized animals. Whereas *H. felis* challenge similarly stimulated the urease-specific serum IgA of rUrease-immunized mice, these levels constituted about 40% of the serum IgG antibody level (not shown). The highest level of serum IgA antibody against *H. felis* was measured in mice challenged 6 weeks post-

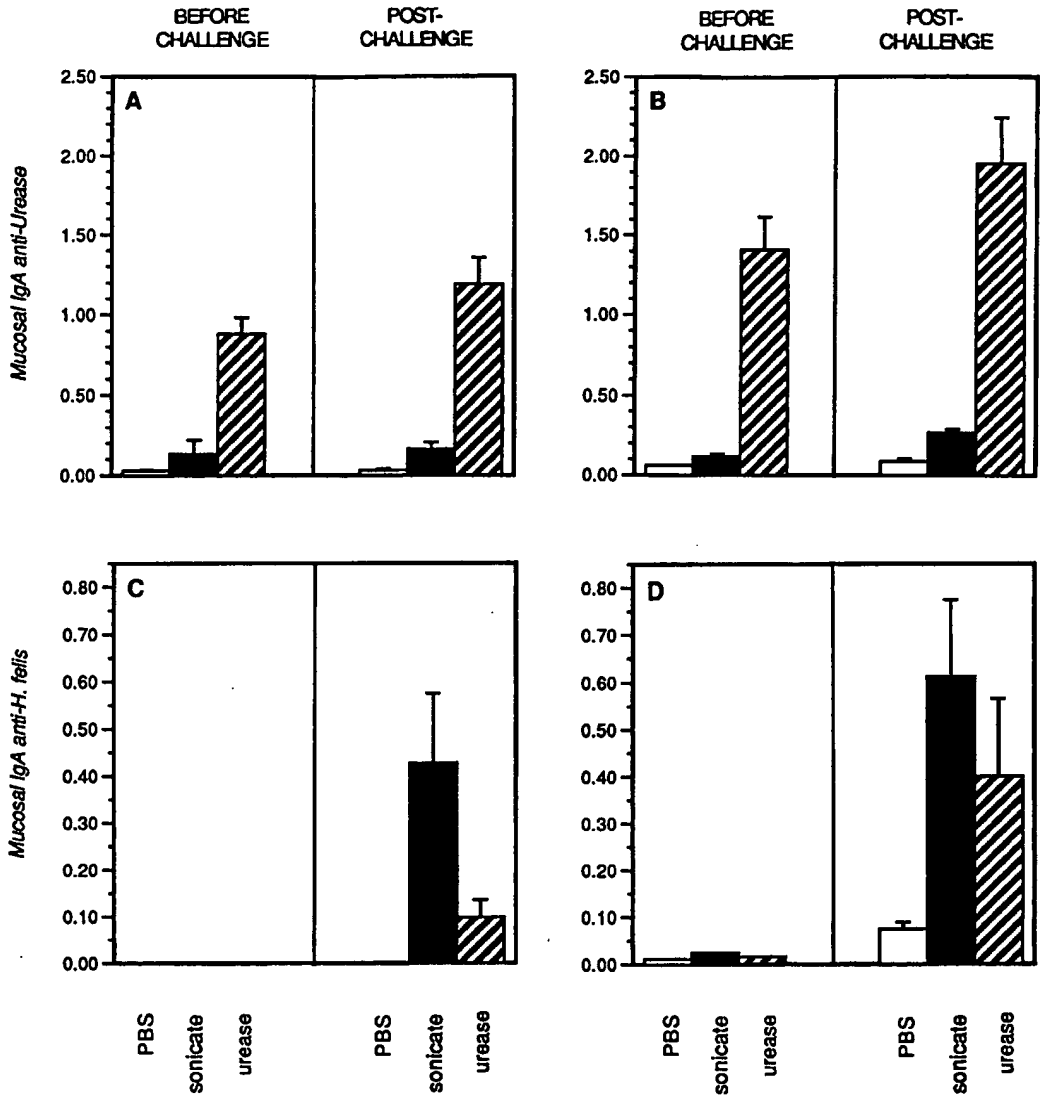

FIG. 3. Secretory IgA antibody levels in the intestinal compartment of Swiss Webster mice immunized orally with PBS (open bars; $n = 4$), *H. felis* sonicate (filled bars; $n = 5$), or *H. pylori* rUrease (striped bars; $n = 5$). The mice were challenged with live *H. felis* 2 weeks (A and C) or 6 weeks (B and D) after the last immunization, and the fecal IgA antibody levels against urease (A and B) or *H. felis* (C and D) were quantitated by ELISA. The bars show the mean optical density at 405 nm for each group, and the brackets enclose 1 standard error of the mean.

rUrease immunization (optical density at 405 nm = 0.337 ± 0.117). The magnitude of both serum IgG and IgA antibody responses against rUrease and *H. felis* antigens at the termination of the experiment was greatest in mice immunized with rUrease.

Intestinal antibody responses. The specific fecal IgA antibody response against urease and *H. felis* antigens before and after *H. felis* challenge is shown in Fig. 3. Oral immunization with rUrease generated much greater IgA anti-urease antibody levels than did immunization with *H. felis* sonicate antigens. Whereas all mice immunized with rUrease developed anti-urease IgA, fecal urease-specific antibody responses were low (optical density at 405 nm < 0.16) or were not measurable in groups of mice immunized with *H. felis* sonicate. Challenge of rUrease-immunized mice with *H. felis* 2 or 6 weeks postimmunization increased the urease-specific intestinal IgA antibody level. However, *H. felis* challenge of PBS-treated control mice or *H. felis* sonicate-immunized mice had little effect on the intestinal IgA anti-urease antibody concentration (Fig. 3A and B). Although the fecal IgA antibody levels against *H. felis* resulting from oral delivery of antigen were very low, oral immunization of mice with rUrease or with *H. felis* antigens effectively primed the mucosal IgA compartment, as shown by the generation of much greater IgA responses in antigen-immunized animals after inoculation with live *H. felis* (Fig. 3).

Histopathology and immunohistopathology of gastric tissue. Inflammatory changes were observed in all mice in both the body and the antral regions in the subglandular portion of the mucosa and frequently extended into the underlying submucosa. The cell infiltrates were characterized as active chronic inflammation and were present as multifocal, relatively diffuse aggregates of mononuclear leukocytes and neutrophils.

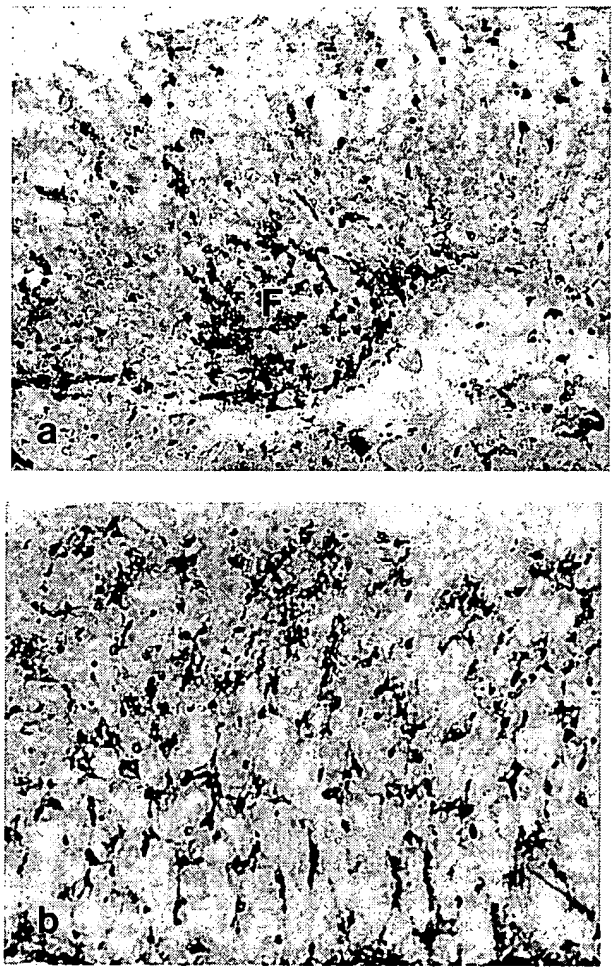

FIG. 4. Immunohistochemical localization of infiltrating Thy1.2$^+$ T cells in cross sections of gastric mucosa approximately 40 days post-oral inoculation with *H. felis*. (a) Gastric tissue from *H. felis*-infected mice immunized with PBS showing lymphoid follicles (F) delimited by scattered mucosal Thy1.2$^+$ T cells. (b) The gastric mucosa from rUrease-immunized mice subsequently challenged with *H. felis* exhibiting predominant clusters of mucosal and submucosal Thy1.2$^+$ T cells. Magnification, ×500.

Immunohistochemical analyses of PBS-treated control animals challenged with *H. felis* revealed multifocal CD45R$^+$ and IgM$^+$ B cells assembled into mucosal and submucosal lymphoid follicles frequently surrounded by aggregates of Thy1.2$^+$ T cells (Fig. 4). In contrast, the gastric tissue from rUrease- or *H. felis* sonicate-immunized animals contained scattered CD45R$^+$ B cells and occasional mucosal follicles but exhibited mild to moderate infiltration by Thy1.2$^+$ T cells distributed as single cells or as clusters in the gastric mucosa and submucosa (Fig. 4 and Table 2). All mice showed a similar distribution of CD3$^+$ CD4$^+$ αβTCR-positive T cells. CD8$^+$ or γδ T cells were infrequently observed in gastric mucosae. Populations of CD5$^+$, CD11b$^+$, and IgA$^+$ cells occurred as isolated cells or as small clusters of cells in the gastric pits, lamina propria, and epithelium. The gastric epithelium and infiltrating mononuclear leukocytes were substantially I-A^{p+} (Table 2). The architectural compositions of cell populations infiltrating gastric tissue were similar whether mice were challenged with *H. felis* 2 or 6 weeks after the last oral immunization.

TABLE 2. Relative phenotypic distributions of mononuclear leukocytes infiltrating gastric tissue after oral immunization and challenge with *H. felis*

Antigen structure or CD designation	Findings for experimental group immunized with[a]:		
	PBS	*H. felis* sonicate	rUrease
Thy1.2	+	+	+
CD3	+	+	+
CD4	+	+	+
CD5	+	+	+
CD8	−[b]	−[b]	−[b]
αβTCR	+	+	+
γδTCR	−[b]	−[b]	−[b]
CD45R	+++	+	+
IgA	+[c]	+[d]	+[d]
IgM	++[e]	+[d]	+[d]
CD11b	+	+	+
CD49d (VLA-4)	+	+	+
CD62L (L-selectin)	−[b]	−[b]	−[b]
I-A^p	+++[f]	+++[f]	+++[f]

[a] The degree of gastric infiltration and/or expression of antigens was scored as mild (+), moderate (++), or severe (+++).
[b] Few scattered cells in mucosa.
[c] Weakly stained cells in follicle coronas.
[d] Scattered cells in mucosa, lamina propria, and epithelium.
[e] Densely labeled cells in lymphoid follicles.
[f] Positive reaction in epithelium and infiltrating leukocytes.

DISCUSSION

In the present study, we have shown that mice orally immunized with *H. pylori*-derived rUrease were protected from infection upon challenge with living *H. felis* organisms. Oral immunization with rUrease resulted in the generation of secretory IgA antibody, and high levels of intestinal IgA antibody against urease were associated with protection of mice against infection when challenged with living *H. felis*. The high degree of conservation at the amino acid level between *H. pylori* and *H. felis* urease (11) may account for the immunological basis of cross-protection with UreB subunit containing protective epitopes (11a, 26). The ability of rUrease antigen to confer protection in immunized mice against infection with *H. felis* was similar to that afforded by *H. felis* sonicate antigens, as shown in this study and others (2, 6, 22). However, while the protective effect of oral immunization with *H. felis* sonicate antigens has been observed in mice challenged with *H. felis* 3 to 14 days after the last immunization (2, 22), we have found the protective response to last up to 6 weeks postimmunization. The findings reported herein are consistent with previous studies showing induction of mucosal IgA and absence of infection in mice immunized with *H. felis* sonicate (6). Furthermore, the present observations indicate that intestinal anti-urease IgA antibody activated by immunization with rUrease may be required to interfere with the establishment of *H. felis* infection and suggest that low levels of urease-specific IgA, as assayed in *H. felis* sonicate-immunized mice, may be protective as well. However, it is not clear whether IgA antibody responses directed against *H. felis* surface structures other than urease may also interfere with colonization. Detailed analyses of antibody responses to *H. felis* surface antigens in protected mice and studies with urease-negative *Helicobacter* mutants (8, 36) may help answer this question. Because IgA may function in the mucosal environment by inhibition of microbial adherence to epithelial cells (39), the mucosal anti-urease IgA antibody response generated in animals immunized with rUrease or *H.*

felis sonicate would have the potential to interfere with the *H. felis*-gastric epithelium interactions which result in colonization. The findings that high levels of IgG antibody induced by parenteral immunization did not protect against infection (3, 9) and that an orally administered anti-*H. felis* MAb protected mice against *H. felis* infection (6) support an important role for local IgA antibody in the prevention of infection in target gastric tissue. The ability of oral immunization to prime the mucosal compartment for a greater IgA antibody response upon deliberate challenge with live *H. felis*, presumably via recognition of urease epitopes displayed at the *H. felis* surface or present as luminal antigen, suggests that protective levels of IgA antibody may be generated in uninfected, immunized hosts as a function of reexposure to the organism.

The continued presence of *H. felis* in the gastric mucosa of PBS-treated mice, as shown in this study, and in gastric tissue of chronically infected mice (12) may signal persistent antigenic stimulation by *H. felis* which gives rise to gastric germinal-center reactions, whereas the reduction or absence of gastric lymphoid follicles in immunized animals may reflect clearance of the organisms and down-regulation of B-cell function. The observation of regression of B-cell gastric lymphoma after eradication of *H. pylori* (40) supports the notion that formation of organized lymphoid tissue in gastric mucosa may be antigen driven. Whether the gastric epithelium overlying mucosal follicles harbors M cells specialized for antigen uptake found in intestinal lymphoid tissues (29) or whether the follicular architecture is maintained by stimulation with luminal *H. felis* antigens which gain access to gastric mucosa is not known at present. However, the finding of scattered $CD45R^+$ B cells and IgM^+ and IgA^+ B cells in gastric tissue of protected animals raises the possibility that oral immunization with rUrease may result in the accumulation and proliferation in gastric tissue of IgA antibody-secreting cells activated in mucosa-associated lymphoid tissue (38).

Recent studies have suggested that T-cell populations from infected hosts are sequestered into gastric mucosa (20) and may regulate local B-cell function and IgA antibody secretion (12). The finding of discrete T-cell populations in murine gastric mucosa after inoculation with live *H. felis* suggests the recruitment and/or local proliferation of T cells dominated by the $CD3^+$, $CD4^+$ $CD8^-$, and $\alpha\beta TCR^+$ phenotypes. Although the gastric TCR specificities for *H. felis* antigens in infected or protected mice have not been probed, recent findings have shown the antigen-specific activation of gastric T cells (19). That gastric $CD4^+$ $CD8^-$ T cells may play a role in local IgA antibody production is also suggested by findings of increased gastric IgA antibody after oral immunization with *H. felis* antigens (6) or with rUrease (28).

Previous studies have found up-regulation of class II major histocompatibility complex expression by gastric epithelial cells in infected patients (10). Whereas I-A antigen expression by gastric epithelial cells and infiltrating mononuclear leukocytes was shown in the present work, these observations suggest that interaction with, but not necessarily colonization by, *H. felis* may be sufficient for induction of I-A, since no differences in the immunohistochemical expression of I-A antigen were seen between infected and protected mice. While the effects of oral immunization on the activation of immunological effector functions by gastric T cells and B cells are not well understood, the current observations indicate that oral immunization with rUrease antigen interferes with the establishment of infection with *H. felis*.

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REFERENCES

1. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161**:626–633.
2. **Chen, M., A. Lee, and S. L. Hazell.** 1992. Immunisation against *Helicobacter* infection in a mouse *Helicobacter felis* model. Lancet **339**:1120–1121.
3. **Chen, M., A. Lee, and S. L. Hazell.** 1993. Immunisation against gastric infection with *Helicobacter* species: first step in the prophylaxis of gastric cancer. Zentralbl. Bakteriol. **280**:155–165.
4. **Clayton, C. L., B. W. Wren, P. Mullany, A. Topping, and S. Tabaqchali.** 1989. Molecular cloning and expression of *Campylobacter pylori* species-specific antigens in *Escherichia coli* K-12. Infect. Immun. **57**:623–629.
5. **Crabtree, J. E., T. M. Shallcross, J. I. Wyatt, J. D. Taylor, R. V. Heatley, B. J. Rathbone, and M. S. Losowsky.** 1991. Mucosal humoral immune response to *Helicobacter pylori* in patients with duodenitis. Dig. Dis. Sci. **36**:1266–1273.
6. **Czinn, S. J., A. Cai, and J. G. Nedrud.** 1993. Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. Vaccine **11**:637–642.
7. **Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser.** 1990. Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265**:9464–9469.
8. **Eaton, K. A., C. L. Brooks, C. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59**:2470–2575.
9. **Eaton, K. A., and S. Krakowka.** 1992. Chronic active gastritis due to *Helicobacter pylori* in immunized gnotobiotic piglets. Gastroenterology **103**:1580–1586.
10. **Engstrand, L., A. Scheynius, C. Pahlson, L. Grimelius, A. Schwan, and S. Gustavsson.** 1989. Association of *Campylobacter pylori* with induced expression of class II transplantation antigens on gastric epithelial cells. Infect. Immun. **57**:827–832.
11. **Ferrero, R. L., and A. Labigne.** 1993. Cloning, expression and sequencing of *Helicobacter felis* urease genes. Mol. Microbiol. **9**:323–333.
11a. **Ferrero, R. L., J.-M. Thieberge, M. Huerre, and A. Labigne.** 1994. Recombinant antigens prepared from the urease subunits of *Helicobacter* spp.: evidence of protection in a mouse model of gastric infection. Infect. Immun. **62**:4981–4989.
12. **Fox, J. G., M. Blanco, J. C. Murphy, N. S. Taylor, A. Lee, Z. Kabok, and J. Pappo.** 1993. Local and systemic immune responses in murine *Helicobacter felis* active chronic gastritis. Infect. Immun. **61**:2309–2315.
13. **Fox, J. G., A. Lee, G. Otto, N. S. Taylor, and J. Murphy.** 1991. *Helicobacter felis* gastritis in gnotobiotic rats: an animal model of *Helicobacter pylori* gastritis. Infect. Immun. **59**:785–791.
14. **Haneberg, B., D. Kendall, H. M. Amerongen, F. M. Apter, J.-P. Kraehenbuhl, and M. R. Neutra.** 1994. Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect. Immun. **62**:15–23.
15. **Hawtin, P. R., A. R. Stacey, and D. G. Newell.** 1990. Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies. J. Gen. Microbiol. **136**:1995–2000.
16. **Hazell, S. L., T. J. Brody, A. Gal, and A. Lee.** 1987. *Campylobacter pyloris* gastritis. I. Detection of urease as a marker of bacterial colonization and gastritis. Am. J. Gastroenterol. **82**:292–296.
17. **Hu, L.-T., P. A. Foxall, R. Russell, and H. L. T. Mobley.** 1992. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by *ureA* and *ureB*. Infect. Immun. **60**:2657–2666.
18. **Hu, L.-T., and H. L. T. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. **58**:992–998.
19. **Hussell, T., P. G. Isaacson, J. E. Crabtree, and J. Spenser.** 1993. The response of cells from low grade B cell gastric lymphomas of mucosa associated lymphoid tissue to *Helicobacter pylori*. Lancet **342**:571–574.
20. **Karttunen, R.** 1991. Blood lymphocyte proliferation, cytokine secretion and appearance of T cells with activation surface markers in cultures with *Helicobacter pylori*. Comparison of the response of subjects with and without antibodies to *H. pylori*. Clin. Exp. Immunol. **83**:396–400.
21. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173**:1920–1931.
22. **Lee, A., and M. Chen.** 1994. Successful immunization against gastric infection with *Helicobacter* species: use of a cholera toxin B-subunit–whole-cell vaccine. Infect. Immun. **62**:3594–3597.
23. **Lee, A., J. G. Fox, G. Otto, and J. Murphy.** 1990. A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology **99**:1315–1323.
24. **Lee, C. K., R. Weltzin, W. D. Thomas, H. Kleanthous, T. H. Ermak, G.**

Soman, J. E. Hill, S. K. Ackerman, and T. P. Monath. Oral immunization with recombinant *Helicobacter pylori* urease induces secretory IgA antibodies and protects mice from challenge with *Helicobacter felis*, submitted for publication.

25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Michetti, P., I. Corthesy-Theulaz, C. Davin, R. Haas, A.-C. Vaney, M. Heitz, J. Bille, J.-P. Kraehenbuhl, E. Saraga, and A. L. Blum.** 1994. Immunization of BALB/c mice against *Helicobacter felis* infection with *Helicobacter pylori* urease. Gastroenterology **107**:1002–1011.
27. **Nomura, A., G. N. Stemmermann, P. H. Chyou, I. Kato, P. G. Perez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. **325**:1132–1136.
28. **Pappo, J., and L. Attardo.** Unpublished observations.
29. **Pappo, J., and T. H. Ermak.** 1989. Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. Clin. Exp. Immunol. **76**:144–148.
30. **Perez-Perez, G. I., B. M. Dworkin, J. E. Chodos, and M. J. Blaser.** 1988. *Campylobacter pylori* antibodies in humans. Ann. Intern. Med. **109**:11–17.
31. **Stohrer, R., and J. F. Kearney.** 1983. Fine idiotype analysis of B cell precursors in the T-dependent and T-independent responses to α1→3 dextran in BALB/c mice. J. Exp. Med. **158**:2081–2094.
32. **Stolte, M., and S. Eidt.** 1989. Lymphoid follicles in antral mucosa: immune response to *Campylobacter pylori*? J. Clin. Pathol. **42**:1269–1271.
33. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
34. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
35. **Trejdosiewicz, L. K.** 1992. Intraepithelial lymphocytes and lymphoepithelial interactions in the human gastrointestinal mucosa. Immunol. Lett. **32**:13–20.
36. **Tsuda, M., M. Karita, M. G. Morshed, K. Okita, and T. Nakazawa.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect. Immun. **62**:3586–3589.
37. **von Wullfen, H., J. Heesemann, G. H. Butzow, T. Lonong, and R. Laufs.** 1986. Detection of *Campylobacter pyloridis* in patients with antrum gastritis and peptic ulcers by culture, complement fixation test, and immunoblot. J. Clin. Microbiol. **24**:716–720.
38. **Weisz-Carrington, P., M. E. Roux, M. McWilliams, J. M. Phillips-Quagliata, and M. E. Lamm.** 1979. Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: evidence for a generalized secretory immune system. J. Immunol. **123**:1705–1708.
39. **Williams, R. C., and R. J. Gibbons.** 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science **177**:697–699.
40. **Wotherspoon, A. C., C. Doglioni, T. C. Diss, L. Pan, A. Moschini, M. de Boni, and P. Isaacson.** 1993. Regression of primary low grade B cell gastric lymphoma of mucosa-associated lymphoid tissue after eradication of *Helicobacter pylori*. Lancet **342**:575–577.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	US 5 843 460 A (SUERBAUM SEBASTIN ET AL) 1 December 1998 (1998-12-01) * column 2, line 13 - line 47 * * claims 1-10; figure 4 *	1-10	C12N9/80 C07K14/205 C07K16/12 C12Q1/68 A61K39/106 A61K48/00 G01N33/53 G01N33/68
X	MYERS G A ET AL: "Oral immunization with recombinant Helicobacter pylori urease confers long-lasting immunity against Helicobacter felis infection" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 17, no. 11-12, March 1999 (1999-03), pages 1394-1403, XP004158267 ISSN: 0264-410X * page 1401; table 1 *	1-10	
X	BLANCHARD T G ET AL: "UREASE-SPECIFIC MONOCLONAL ANTIBODIES PREVENT HELICOBACTER FELIS INFECTION IN MICE" INFECTION AND IMMUNITY,US,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, vol. 63, no. 4, 1 April 1995 (1995-04-01), pages 1394-1399, XP000670120 ISSN: 0019-9567 * page 1396, column 2; figures 1-3,6 *	1-10	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N C12Q C07K A61K G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15 December 2000	van Klompenburg, W

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